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BIOREMEDIATION
FACTORS INFLUENCING METAL ION TOXICITY
IN A TOLUENE-SELECTED BACTERIAL POPULATION

Thesis

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Bioremediation:

Factors Influencing Metal Ion Toxicity in a Toluene-Selected Bacterial Population

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BIOREMEDIATION:

FACTORS INFLUENCING METAL ION TOXICITY IN A TOLUENE-SELECTED BACTERIAL POPULATION

THESIS

Presented to the Faculty of the School of Engineering
of the Air Force Institute of Technology
Air University In Partial Fulfillment of the
Requirements for the Degree of
Masters of Science in Engineering and Environmental Management

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Scott Hansen

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Abstract

This research was a general phenomenalistic overview of the effects of metals on the respiration rates of a toluene selected microbe culture.

The metals studied were copper, zinc, cobalt, manganese and iron. Relative inhibition, Cu>Fe>Zn≈Co>Mn, corresponded to hydrogen phosphate binding strengths. Inhibition was found to be pH dependent; it increased with increasing pH and was shown to correlate to an increased adsorption of metal onto the cell.

Zn, Cu, and Co toxicities were shown to decrease with increasing magnesium concentrations. This decrease was linked to increased magnesium adsorption and decreased metal adsorption. The magnitude of effect was related to Mg competitiveness.

Sterile plating techniques were used to determine metal lethality. Results did not correspond to respiration results; however, it was demonstrated that copper's effect was lethal while zinc's was inhibitory.

Increasing metal concentrations cause toxicity to increases at a decreasing rate only so long as the metal remains in solution. Metals as precipitates are no longer available to adsorb to the cell and do not affect toxicity.

Toxicity was shown to be related to metal availability and how well it competed with other cations. Toxicity also depended on cell-ligand competition and how strongly the metal bound itself to the cell.

I.0 Introduction:

1.1 Background

This paper will explore the effects of metals on the metabolic rate of a toluene selected microorganism culture. It is a general phenomenological overview of selected metals and the factors effecting metal induced metabolic inhibition. An attempt will be made to correlate the inhibition of metals to their adsorption on microorganisms' cell walls; concentration effects, pH effects, competitive cation effects, and ligand effects will also be explored. Its purpose is to better understand the effects of metals on microbial activity, especially the activity related to the bioremediation of hazardous waste sites.

1.1.1 Bioremediation

For the purpose of this paper, bioremediation is defined as the "use of biological agents to reclaim soils and waters polluted by substances hazardous to human health and/or the environment" (Atlas and Bartha, 1993:534). In addition, the definition will be expanded to include biofilters which use biological agents to prevent the environmental release of pollutants from industrial operations.

Bioremediation has been gaining popularity world wide as an effective means of cleaning up contaminated sites and preventing the release of contaminants. It is seen as a "green" technology (Atlas, 1995:32) because in most cases it mineralizes hydrocarbon contaminants without the long term liability and residual health risk of land filling or incineration. In 1990 it was a 40 billion dollar per year industry, by the year 2000 it is

expected to grow to a 75 billion dollars per year (Atlas, 1995:32). As it gains popularity its world wide market continues to grow.

The expected growth is due in part to the nearly comprehensive ability of microorganisms to degrade environmental pollutants, particularly hydrocarbons, and to concentrate other materials, i.e. metals in waste streams. The ability to degrade most chemicals already exists in the *in situ* population. By the proper application of nutrients and /or oxygen populations of degraders can be made to flourish.

In those cases where native organisms cannot degrade the pollutants, specialty organisms that can grow under especially harsh temperature, pH or chemical conditions can be introduced into the environment (Haigler and others, 1992:2239), (Spain, and Gibson, 1989:2649), and (Malachowsky and others, 1994:542). In Japan, researchers have isolated a strain of *Pseudomonas* that can grow in solvents containing over 50% toluene (Atlas, 1995:32). Still further work is under way to genetically alter microorganisms to combine the best features of several microorganisms to create "super" bugs. This work however, does not currently play a role in bioremediation because of regulatory requirements and fear that these microorganisms will transfer their genetic alterations to the natural populations.

Although microbial bioremediation is generally thought of as a means of remediating hydrocarbons, research has shown that microbes are capable of adsorbing great quantities of metals and have the potential to remediate heavy metal contamination (McLean and others, 1994:472; Mullen and others, 1989:3143; Flemming and others, 1990:3219). An example of microbial bioremediation currently being used on heavy

metals involves the use of sulfate reducing bacteria under anaerobic conditions to immobilize zinc and cadmium from contaminated ground water. In this remediation effort, heavy metal concentrations in the effluent from a pump and treat process are reduced to just a few parts per billion (Atlas, 1995:38). In other research, *Pseudomonas* bacterium were observed reducing total petroleum hydrocarbons from 4700 ppm to 160 ppm, while decreasing soluble lead levels from 240 ppm to 83 ppm (McCullough and Dagdigian, 1993:277). Thus bioremediation has shown the potential to be used in the treatment of both organic and inorganic wastes

In the United States there are more than 1,200 superfund that have the potential to be bioremediated. Bioremediation can be at least ten times cheaper than other remediation efforts, making it an attractive alternative to the physical removal and subsequent destruction or relocation of pollutants (Atlas, 1995:35). A significant number of these superfund sites also possess heavy metal contamination, (fully 1/6 of the sites are contaminated with lead) (McCullough and Dagdigian, 1993:265). This complicates the remediation effort, remediation optimized for one purpose may fail to improve or may even worsen the contamination caused by another waste type. Creating an environment that cultivates a population that will mineralize all the organics and immobilize the inorganics is not always biologically or financially possible.

In addition to cleaning up spill sites, bioremediation is being considered in many European countries and Japan as a pollution control technique for the end of pipe treatment for both air and industrial waste water streams. The Netherlands for instance

has over 200 biofilters in use (Atlas, 1995:32). In Japan, research is under way to create biofilters that produce energy sources as alternatives to fossil fuels (Atlas, 1995:40).

1.2 The problem

A wide variety of treatment options exist for soils contaminated with hydrocarbons or metals, of which bioremediation is only one option (sometimes, but not always, the best one). These options include thermal destruction, low temperature thermal desorption, soil washing/solvent extraction, dechlorination, immobilization and bioremediation. Each works well in specific situations, and with one type of waste; however, often many types of wastes exist simultaneously. Sites containing both heavy metals and hydrocarbons existing together, usually cannot be successfully treated by one treatment (McCullough and Dagdigian, 1993:265). These situations often result in the use of a multiple treatment process or remediation train, resulting in higher clean-up costs.

Hydrocarbons in the environment can be very mobile, threatening to contaminate ground water supplies; bioremediation is particularly well suited to the remediation of these sites. Metals in the environment tend to be fairly immobile; they are quickly chelated to organic matter or the soil matrix. In the presence of certain types of hydrocarbons, the mobility of the metals can be greatly increased. Metals may partition into the organic phase and be carried along with the contaminant thus increasing the metal contamination of the site. The metals may decrease the rate of the biodegradation allowing the hydrocarbon plume to advance further. The two types of waste may act

synergistically to increase the extent of environmental impact (McCullough and Dagdigian, 1993:269).

One popular process for the remediation of organic and inorganic waste sites is the use of bioremediation followed by soil stabilization (Vipulanandan and others, 1993:537). Soil stabilization effectively immobilizes the metals but it is ineffective in containing the hydrocarbons; bioremediation will degrade the hydrocarbons but will not significantly decrease the mobility of the metals, thus both processes must be used. In cases of sites containing both organic and inorganic pollutants, the design of remediation efforts will require knowledge of hydrocarbon-metal interactions and knowledge of the metal effects on bioremediation.

The problem is that researchers do not understand all the interactions between organic and inorganic pollutants. In order to more effectively design remediation efforts these interactions must be studied. The role of research today is to attempt to understand microbial ecology and the biochemical processes that they entail. The researcher's ultimate goal is to be able to understand and control the factors associated with bioremediation so that they might be optimized.

1.3 Past Research Focus

The sum of past research, although substantial, is still incomplete. Factors affecting the bioremediation are still not well understood. One area of concern is the effect of metal contamination on bioremediation.

Past research has focused on the effects of one or two factors on biodegradation. Much work has been done on the effects of environmental parameters such as pH, dissolved oxygen (DO), nutrients and temperature on bioremediation (Knaebel and others, 1994:4500; Swindoll and others, 1988:212; Tatara and others, 1993:2126; Jones and Alexander, 1988:2177; Lewis and others, 1986:598). This work has primarily focused on *in situ* bioremediation and the conditions that can be altered to improve degradation rates. Additionally, great effort has been invested in isolating microorganisms that degrade a wide variety of organic compounds (Malachowsky and others, 1993:542; Spain and Gibson, 1988:1399; Pettigrew and others, 1991:257; Spain and others, 1989:2648). Work has included isolating organisms that degrade particularly recalcitrant compounds as well as organisms that degrade a wide variety of compounds. This research has shown that environmental factors do affect biodegradation, biodegradation rates can be enhanced and microorganisms do exist that degrade a wide variety of compounds.

Significant efforts have been made to understand bioremediation kinetics. This research is particularly important as more work is done not only in the design of biofilters and bioreactors, but also *in situ* bioremediation. Current research in degradation kinetics is focusing not only on the degradation of one hydrocarbon, but on the degradation of several hydrocarbons simultaneously (Alverez and Vogel, 1991:2981; Arvin and others, 1989:3221; Pettigrew and others, 1991:157; Haigler and others, 1992:2237). This research has been successful for predicting the degradation of one and two hydrocarbons; however the effect of metals on hydrocarbon degradation kinetics is not plentiful.

The interaction of microorganisms with metals is also being studied. The main focus of this research is to determine the role that microorganisms play in the immobilization of heavy metals in the environmental soil matrix. Most of the work has been accomplished on cell membranes and the mucus layer that surrounds the cells (Sicko-Goad and Staerma, 1979:316; McLean and others, 1994; Beveridge and Muray, 1976:473; Mullen and others, 1989:3143; Darnall and others, 1986:206; Poonan and Clandra, 1990:473; Vymaral, 1987:97; Blake and others 1992:1367; Porta and Ronco, 1993:263). This research has found that microorganisms compete with natural ligands and may adsorb large quantities of metals, possibly permitting their use in metal adsorption remediation.

Research has found that certain physiochemical factors influence the effect of metals on microorganisms, factors such a pH, oxidation potential, inorganic and organic ligands, other cations and water hardness may all influence the effect of metals on microorganisms. Two sources, Beveridge and Doyle, 1989: 53-60 and Babich and Stotzky, 1980: 100-130, provide excellent reviews of the effects of environmental factors on metal toxicity.

There has been little research conducted on the effects of heavy metals on the biodegradation rates of hydrocarbons. The mechanisms for the inhibition of the organisms are not well known. Here appears to be a gap in the knowledge of bioremediation. If *in situ* bioremediation is being considered, the degradation rate of contaminants is necessary to estimate the extent of the contaminant plume as well as the length of time for the clean-up process. Even if the site is not affected by metal

contamination above the background levels, natural concentrations of certain metals can affect degradation rates. If a reactor system is being considered, the size of the system will be determined by the amount of ground water processed and the rate of contaminant degradation. Metal contamination of the water will influence the rate of degradation.

1.4 Objective

The objective of this thesis is to investigate the effects of metal contamination on the rate of cellular metabolism. Toluene was chosen as the substrate of interest because of its ubiquity in the environment and known degradation pathway. Metals have been chosen that are representative of the types of metals that might exist at contaminated sites and because they are not considered hazardous wastes. This thesis will attempt to shed insight into the following areas:

1. What is the effect of changing pH?

   What is the effect of pH on metal ion toxicity?

   Rank order metals according to toxicity

   Is there a correlation between toxicity and metal ion adsorption?

2. What is the effect of magnesium and calcium on metal ion toxicity?

   What is the effect of competitive cations on metal toxicity?

   Is there a correlation between toxicity, toxic metal adsorption and competitive cation ion adsorption?

3. Is the toxicity of a metal related to the size of the microbial population?

4. What are the effects of changing nutrient levels on metal toxicity?

5. What is the effect of chloride ion on metal ions toxicity?

6. What is the effect of varying heavy metal concentrations on the maximum degradation rate?

7. Is toxicity as measured by respirometry a good indicator of metal-induced lethality?

1.5 Scope

The scope of this thesis is meant to be a phenomenalistic overview of the effects of metals on the respiration rate of toluene-selected microbe cultures. Copper, zinc, cobalt, manganese and iron will be studied in an attempt to determine what factors effect metal toxicity and possible causes of toxicity. X-ray fluorescence will be employed to determine metal adsorption to cells; this will be correlated to metal toxicity. In addition, the toxicity mechanism of the heavy metals will be examined. The effects of the exposure on the population size will also be examined.

1.6 Possible Benefits

The possible benefits to this research include an understanding of the toxicity mechanism of heavy metals on microbial populations and an understanding of the physiochemical factors have on microbial respiration. This information can be used in the design of bioreactors for the treatment of contaminated ground water, or the design of biofilters for the treatment of air or water effluent from an industrial process.

II. Literature Review

2.1 Properties of the Substances Chosen

The substrate and metals were chosen because they are ubiquitous and we can use them without having to treat them as hazardous waste. In addition they were chosen because of their extensive use in the literature.

2.1.1 Toluene

Toluene was chosen because its properties and degradation are well known. Toluene is representative of petroleum hydrocarbons EPA waste code D049 (Vipulanandan and others, 1994:537).

2.1.1.1 Properties of Toluene

Toluene, empirical formula C_7H_8, is an aromatic compound that is created by the substitution of a methyl group on to a benzene ring. (LaGrega and others, 1994:1050) Toluene is a naturally occurring compound and is a constituent of crude oil. It is used pervasively throughout the industrial world as a component in fuels, paints, coatings, and as a benzene substitute (Alloway and Ayres, 1993:210). In 1991 the annual US production of toluene was 6.1 thousand tons. That ranked it as one of the highest produced chemical in the country (Alloway and Ayres, 1993:210). In combination with benzene and xylene (BTX compounds) it is produced in gasoline at a combined rate of 24.7×10^9 lb/yr (Young-Sook, 1994:533).

High production rates, high spill rates, and moderate toxicity has lead to its regulation by the EPA. Toluene is a priority pollutant as designated under the Clean Water Act; it is designated a hazardous substance under CERCLA, an Appendix VIII chemical under RCRA, and a HAP (Hazardous Air Pollutant) under the 1991 Clean Air Act (Alloway and Ayres, 1993:210).

Table 2-1

Properties of Toluene

Mol Weight gm/mol	Solubility mg/L	Vapor pressure mmHg/atm	Boiling Point
92.15	515 @ 20 C	20.1 @ 20 C	111 C

(La Grega and others, 1994:1050)

2.1.1.2 Toluene Degradation

Toluene is a hydrocarbon and is degradable primarily under aerobic conditions; molecular oxygen is needed at least for the initial step in the reaction. There are two classes of enzymes that mediate toluene degradation; both classes are termed oxygenases. Those enzymes that fix a single atom of oxygen are termed monooxygenases while those that fix two atoms of oxygen are termed dioxygenases. (Spain and others, 1987:2648) Toluene is degraded aerobically through the use of a toluene dioxygenase system, which tends to be a very non-specific enzyme system.

Toluene is degraded to either a catechol or substituted catechol by one of the following proposed pathways:

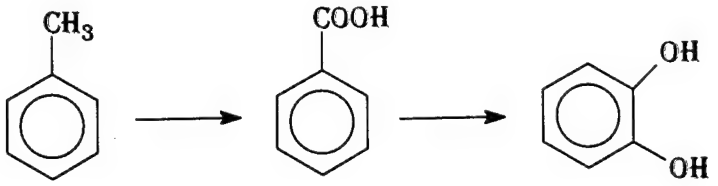


Figure 2-1
Toluene Degradation to Catechol

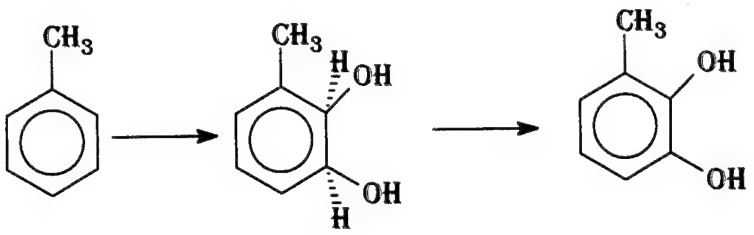


Figure 2-2
Toluene Degradation to Substituted Catechol

The substituted catechol or catechol ring can then be severed one of two ways. It may be broken between the two hydroxyl groups, ortho ring fission, or outside of the hydroxyl groups, meta ring fission. This occurs in the beta-ketoadipate pathway (Figure 2-3)

The by-products of toluene degradation end up in the tricarboxylic acid cycle, also known as the Krebs cycle or the citric acid cycle. It is the terminal reaction in the oxidative respiration of cells and is responsible for the mineralizing the toluene. In addition to its function as the terminal reaction for respiration, the cycle plays an important role in the synthesis of cell material. See Figure 2-4 (Doelle, 1975:380-392)

If a toxic metal is substituted in an enzyme at any point along this process the function of this enzyme may be impaired or halted. This would deprive the cell of energy for metabolic activity and cellular components for growth.

The major metabolic catalyst in the toluene dioxygenase, as well as most other monooxygenase and dioxygenase systems, is iron although in rare cases manganese will sufficise (Beveridge and Doyle, 1989:185). Iron has been isolated in enzyme systems for benzoate, dioxygenase, benzene dioxygenase, and naphthalene dioxygenase. The toluene dioxygenase system is a multi-component system that uses a mini electron transport chain to oxidize and reduce pyridine nucleotide, and ultimately to pass electrons to oxygen for activation (Beveridge and Doyle, 1989:185).

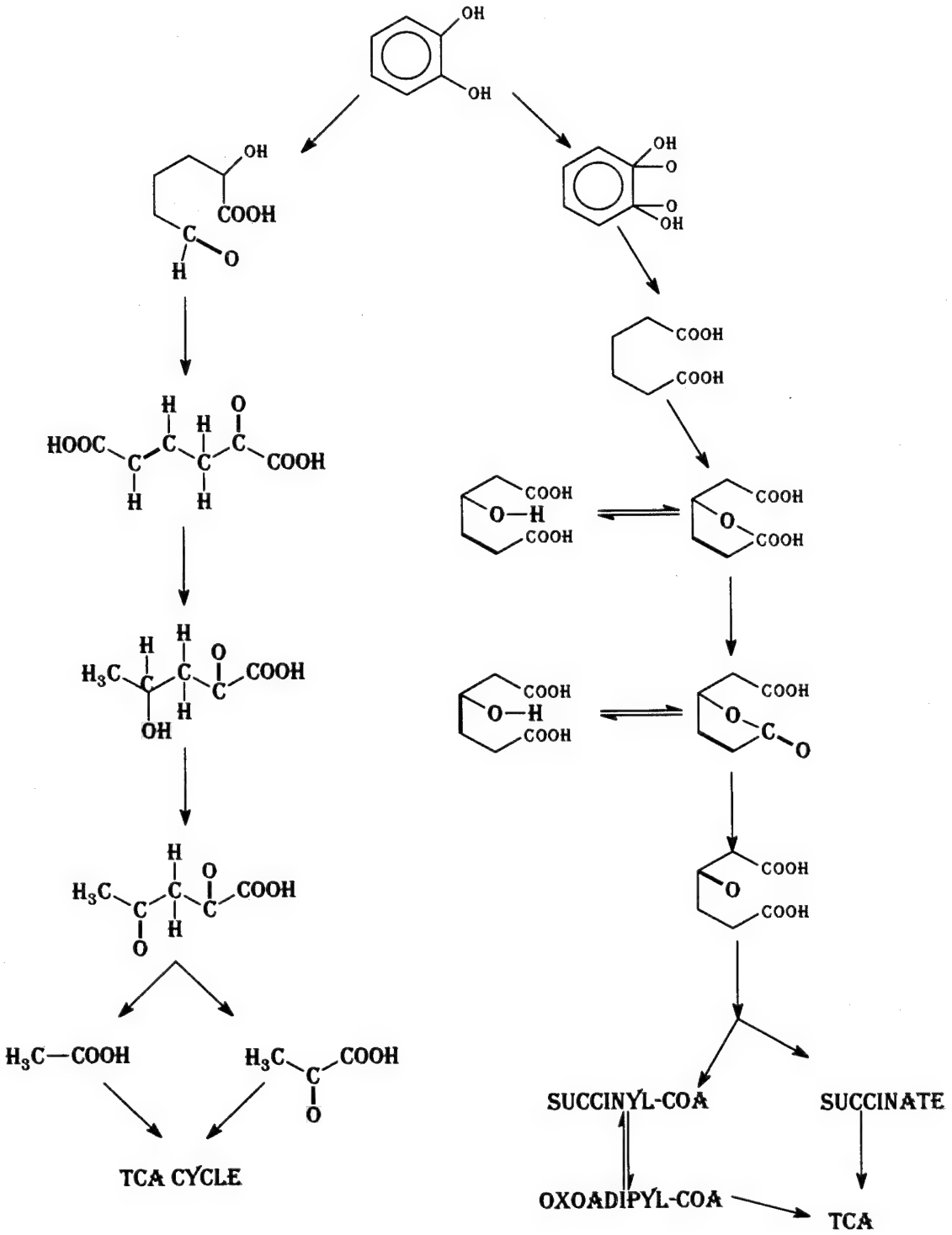


Figure 2-3

Beta-ketoadipate Pathway

(Doelle, 1975:330)

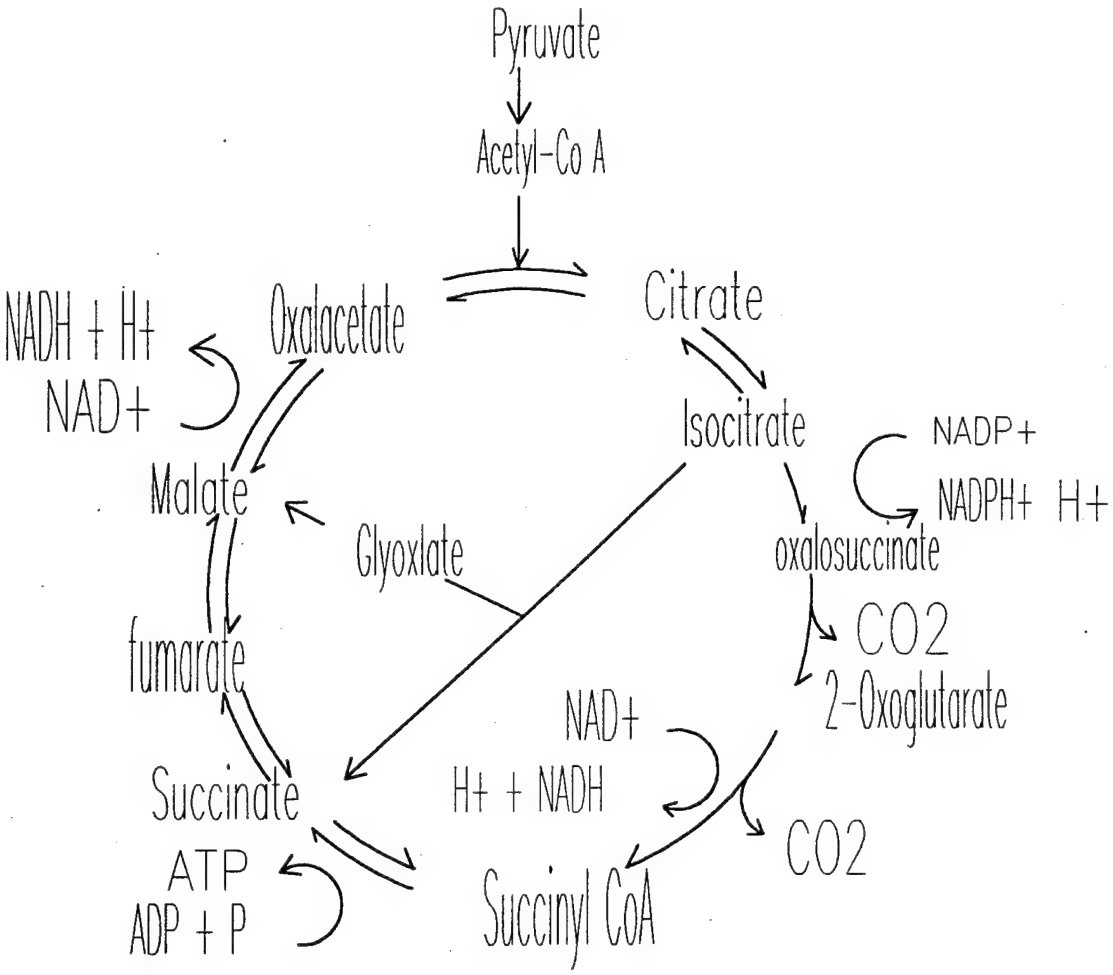


Figure 2-4
Tricarboxylic Acid Cycle (TCA)
(Doelle, 1975:381)

2.1.2 Metals

Metals are those elements whose compounds form positive cations when in solution and whose oxides form hydroxides rather than acids with water (Beveridge and Doyle, 1989:32). The next three sections will present, the increase in metals due to

anthropomorphic pollution, the properties of the metals studied, and several methods for metal classification.

2.1.2.1 Metal Pollution

For thousands of year humans have been extracting elements from the earth, a portion of these metals have been released and redistributed into the biosphere. This problem has escalated since the start of the industrial revolution (Ochiai, 1995:479). Figure 2-5 shows an estimation of increased environmental metallic concentrations due to anthropogenic discharge (Ochiai, 1995:479). Values greater than 1 indicate increased concentrations due to man's activities. For example, environmental concentrations of selenium are roughly 20 times greater than would have been present if no human activity had occurred. It is possible that some of these anthropogenic metals are reducing the primary production of the biota.

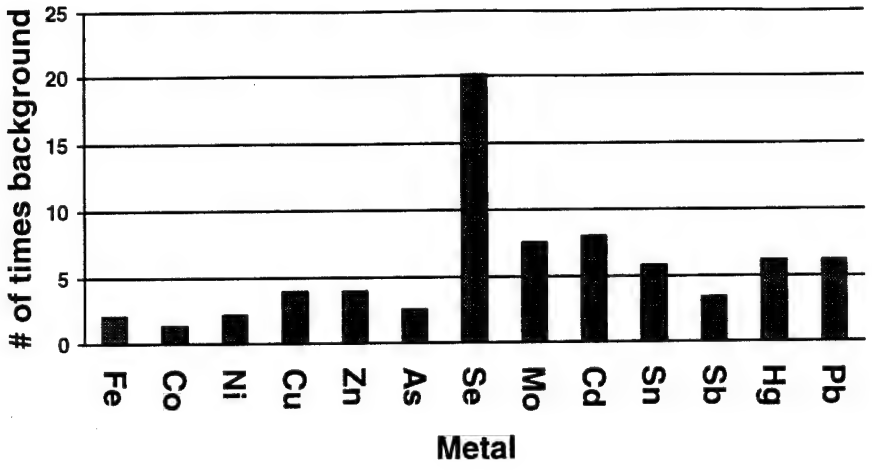


Figure 2-5
Excess burden of metals on the biosphere
(Ochiai, 1995:479)
Numbers greater than 1 indicate human pollution

2.1.2.2 Properties of metals

The following is a list of properties of for the metals used in this thesis. These properties will become important in the evaluation of experiment results to explain possible differences in toxicities and other observed phenomena.

2.1.2.3 Metal Classification

Metals have been classified according to differing properties; these classifications can be used to help predict toxicity. This section will consider three classification schemes; Hard / Soft, Class A / Class B metals, and ligand preference.

There exists in the literature, two metal classification systems that provide useful clues to toxicity. One divides metals into hard acids and soft acids while the other divides them into Class A and Class B metals. These classifications are important; in general toxicity correlated to the softness and class B characteristics of a metal. (Beveridge and Doyle, 1989:36)

2.1.2.3.1 Hard / Soft Acid Classification

The classification of substances into hard or soft Lewis acids or bases was developed by Pearson. The following discussion is a compilation of Pearson's works (Pearson, 1963:3533; Pearson, 1968:581; Pearson, 1968A:643; Pearson, 1973).

Table 2-2

Properties of Metals

Property	Cu	Co	Fe	Mn	Zn	Mg	Ca
Atomic Weight (gm/mol)	63.54	58.93	55.9	54.9	65.39	24.30	40.08
Charge	+2	+2	+3	+2	+2	+2	+2
Ionic Radius (A)	58	75	63	83	60	71	100
Coordination number	8	8	8	8			
Lewis acid type	Border	Border	Hard		Border	Hard	Hard
Ligand Association constants Log K							
PO_4^{3-} M-L						4.8	6.5
M-H-L	16.5	15.5	16.0		15.7	15.3	15.1
M-H_2-L	21.3		22.3		21.2	20.0	21.0
M_3-L_2					35.3	25.2	
M-L-H(s)						18.2	19
M_3-L_2 (s)			36			25.2	
EDTA M-L	20.5	18.1		15.6	18.3	10.6	12.4
M-H-L	23.9	21.5		19.1	21.7	15.1	16
M-O-L	22.6				19.9		
S^{2-} M-L (s)	36.1	21.3		10.5	16.6		
M-L (s)		25.6		13.5	24.7		
OH^- M-L	6.3	4.3	11.8	3.4	5.0	2.56	1.15
M-L_2	11.8	9.2	22.3	5.8	11.1		
M-L_3		10.5	42.7	7.2	13.6		
M-L_4	16.4	34.4	34.4	7.7	14.8		
M_2-L_2	17.7						
M-L_2	19.3	15.7		12.8	15.5 16.8		5.19
M_4-L_4						16.28	
Cl^- M-L	.5	.5	1.5	.6	.4		

(Morel and Hering, 1993:332-341)

Hard Lewis acids are generally small atoms of high positive charge and low polarizibility. They contain no unbound electrons in their valence shell; hence, they have the electron configuration of an inert gas. (Beveridge and Doyle, 1989:33) The electron configuration of a hard acid is stable and not readily deformed in the presence of electromagnetic fields. Water is strongly attracted to hard acid and is not easily displaced from the metal's coordination sphere; it is from this phenomenon that it gets the name hard acid. Hard acids form primarily electrostatic interactions with ligands as opposed to covalent interactions.

Soft acids contain unshared electrons in their outer electron shell. They have a lower electronegativity and are more highly polarizable than soft acids. They primarily participate in covalent bonding with ligands with electrostatic attraction smaller. Soft acids bind ammonia more strongly than water and readily form stable chloro and iodo complexes. Soft acids can also form stable sigma bonded organometallic compounds. For example Hg(II) will bind strongly with such soft bases as S^{2-}, CH_3, and C_2H_5 (Ochiai, 1994:480).

Hard Acids	Soft Acids
Mg^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Co^{3+}	Cd^{2+}, Hg^{2+}

Borderline metals

Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}

Figure 2-6

Partial List of Hard, Soft and Borderline Lewis Acids

(Beveridge and Doyle, 1989:33)

Soft and hard acids have differing affinities for bases. Hard acids prefer the left hand side of the list of bases (hard bases) (Figure 2-7) while soft acid prefer the right hand side (soft bases). It is worth noting that soft acids form more stable complexes with phosphorous and sulfur, major base components in cell membranes and enzyme systems.

F>O>Cl, N>Br>I,S,Se,C>P

Figure 2-7
Ligand preference Hard to Soft

2.1.2.3.2 Class A / Class B Metals

Nueboer and Richardson proposed a system of metal classification based on the equilibrium constants of certain metal-ligand complexes. In this system, ligands are placed into one of three categories Class A, Class B or Borderline ions. Class A ions prefer ligand in the following order F>Cl>Br>I. If the atoms are part of a multi-element ligand the order of preference is O>S>Se, N>As, and O>N>S. Class B ions have the opposite preference sequence: I>Br>Cl>F, Se≈S>O, As>N, and S>N>O (Nieboer and Richardson, 1980:3). Borderline metal are those that exhibit some qualities of each class.

Class A:	Class B:
Be, Na, Mg, K, Ca, Sc, Rb, Sr, Y, Co, Al	Pd, Ag, Ir, Pt, Au, Hg, Ti, B

Borderline:

Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Cd, In, Sb, Pb

Figure 2-8
Class A / Class B metal Classification
(Nieboer and Richardson, 1980:3)

It is more difficult to distinguish borderline metals from class B than it is to distinguish between borderline metal and class A metals. If placed in order according to their closeness to class B character the following order applies (Nieboer and Richardson, 1980:3).

$$Pb^{2+}>Cu^{2+}>Cd^{2+}>Co^{2+}\approx Fe^{2+}>Ni^{2+}>Zn^{2+}>Mn^{2+}$$

Class B metals form water stable organometallic cations that are highly lipid-soluble and therefore readily cross biological membranes and accumulate in cells (Beveridge and Doyle, 1988:36). Class A metals have more ionic nature to their bonds while Class B metals are more covalent. Class B metals form stable methylated molecules in aqueous solution while class A ions do not. The methylated derivations of class B ions as well as inorganic derivations have a high affinity for S-H and S-S groups. S-H and S-S sites play important roles in enzymes and other proteins (Beveridge and Doyle, 1989:36). Methylated forms of Hg bind to nitrogen nucleotide bases in RNA and DNA interfering with the function of these nucleic acids (Beveridge and Doyle, 1989:36).

Generally Class B metals correspond to soft metals and Class A metals correspond to hard metal; however the ordering of the metals is somewhat different.

2.1.2.3.3 Ligand Preference

Class A or hard acid metals have consistently lower stability constants than soft or class B metals. This is due primarily to more ionic nature (weaker bond forming) of the Class A, hard acid metals. Regardless of the ligand used for comparison, the order of stability for divalent cations-ligand complexes remains the same Pd>Cu>Ni>Co>Zn, Cd, Fe> Mn>Mg (Beveridge and Doyle, 1989:37). Crist and others determined the relative strength of adsorption of copper, magnesium, sodium, strontium and zinc to algal cell walls to be Cu>Sr>Zn>Mg>Na thus indicating a variation from covalent to ionic bonding as metals become more Class A/Hard and less Class B/Soft (Beveridge and Doyle, 1989:37).

2.2 Cell Membrane Physiology

In this section the microbial cell membranes for both the gram positive and gram negative microbes, as well as cell envelopes, will be discussed. The cell membrane is the ultimate barrier between the exterior environment and the cytoplasm. It holds the cell together and provides protection. It must allow for the entrance of food, oxygen, water, and essential nutrients, and allow for the exit of waste products. Yet it must also exclude toxic substances.

The membrane is especially necessary for bacteria since they depend on diffusion to get nutrients. Diffusion is maximized by a very large surface area to volume ratio. In

fact, bacteria have the highest surface to volume ratio of any living creature (Beveridge, 1989:147). Owing to this large surface area to volume ratio, bacteria will undoubtedly encounter any toxic metals in the environment and must be able to deal with these incursions.

There is a vast complexity and variation amongst cell membranes. The membrane not only depends on the type of bacteria but also its growth medium (Beveridge, 1989:152). The basic structure of both gram positive and gram negative cell membranes will briefly be discussed.

2.2.1 Gram Positive

Gram positive cell membranes are very simple in comparison to Gram negative. If grown in the presence of phosphate, Gram positive membranes will consist of essentially two substances, peptidoglycan and teichoic acid, with peptidoglycan being the major constituent (Beveridge 1989:150). It is made up of repeating beta linked N-acetylglucosamine-N-acetylmucamyl dimers (Figure 2-9). They are often linked to their neighbors by covalent bonds; the mesh work of intermolecularly bonded dimers is called the peptidoglycan or murein (Figure 2-10). It is the murein to which secondary substances are attached (Beveridge, 1989:150). The Gram positive cell wall contains none of the phospholipids that are common in Gram negative bacteria.

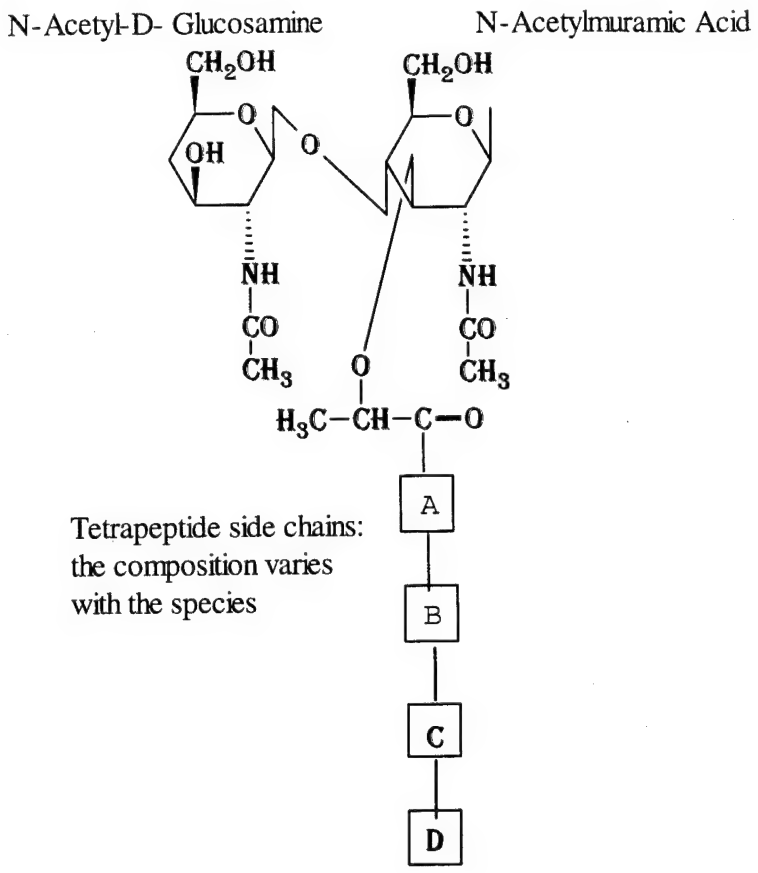


Figure 2- 9

N-acetylglucosamine-N-acetylmucamyl Dimer

(Lehninger, 1982:293)

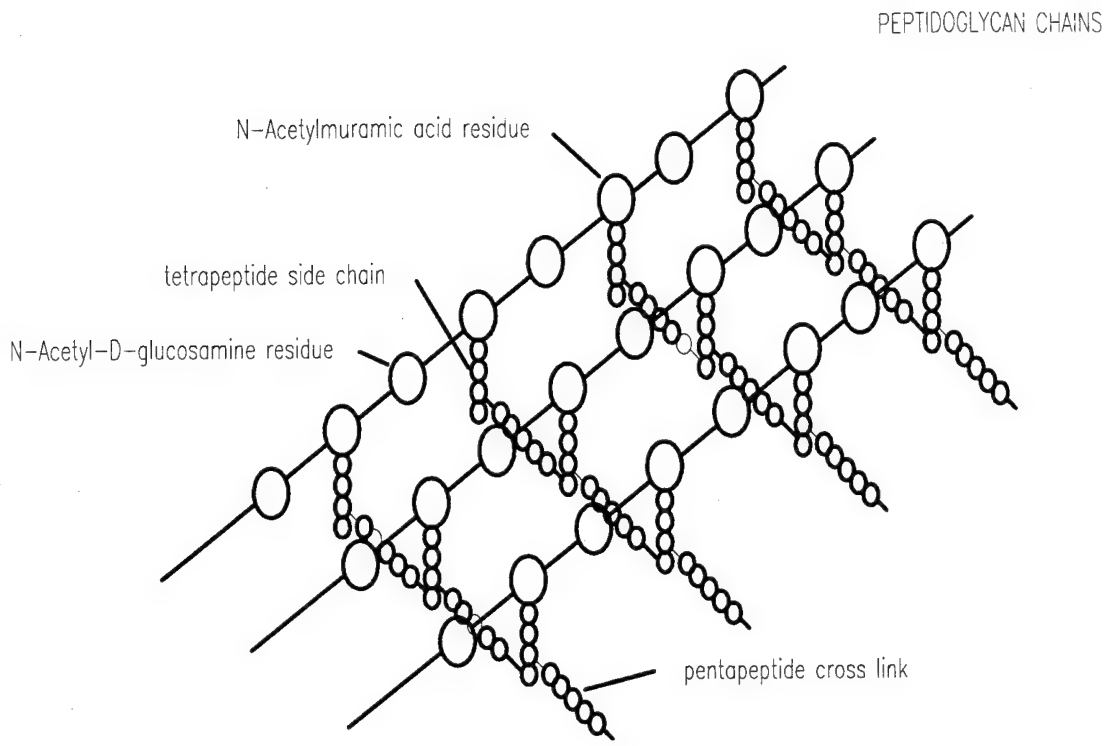


Figure 2-10
Peptidoglycan or Murein
(Lehninger, 1982:293)

In *B. subtilis*, the peptidoglycan can account for 30-50% of the cell wall's weight and its interstrand crosslinkage can involve 20-30% of the muranyl residues. As a result the cell membrane is extremely strong and resilient and carboxylate groups of the peptidoglycan tends to give the organism an overall anionic character (Beveridge, 1989:150-151).

Terchoic acid, the other major component of a gram positive bacterial membrane, is a glycerol based linear polymer joined together by phosphodiester linkages. In *B. subtilis* strain 158, it is covalently bonded to a number of peptidoglycan residue sites. It is believed that the teichoic acid is further stabilized by salt bridging by Mg^{2+} between

the phosphate groups on the teichoic acid and other anionic portions of the muranyl residue (Beveridge 1989:151)

2.2.1 Gram Negative

The cell membrane of the Gram negative bacteria is much more complex than that of the Gram positive. Gram negative bacteria have a peptidoglycan layer that is located between the plasma and outer membranes and is thus not directly exposed to the surrounding environment (Beveridge 1989:151). In *E. coli* and *Pseudomonas aeruginosa* there is only enough peptigoglycan to form a murein layer 1 to 3 molecules thick (Beveridge 1989:151). The bi-lipid polysaccharide membrane resides around the murein layer and in many cases is covalently bonded to the murein (Figure 2-11).

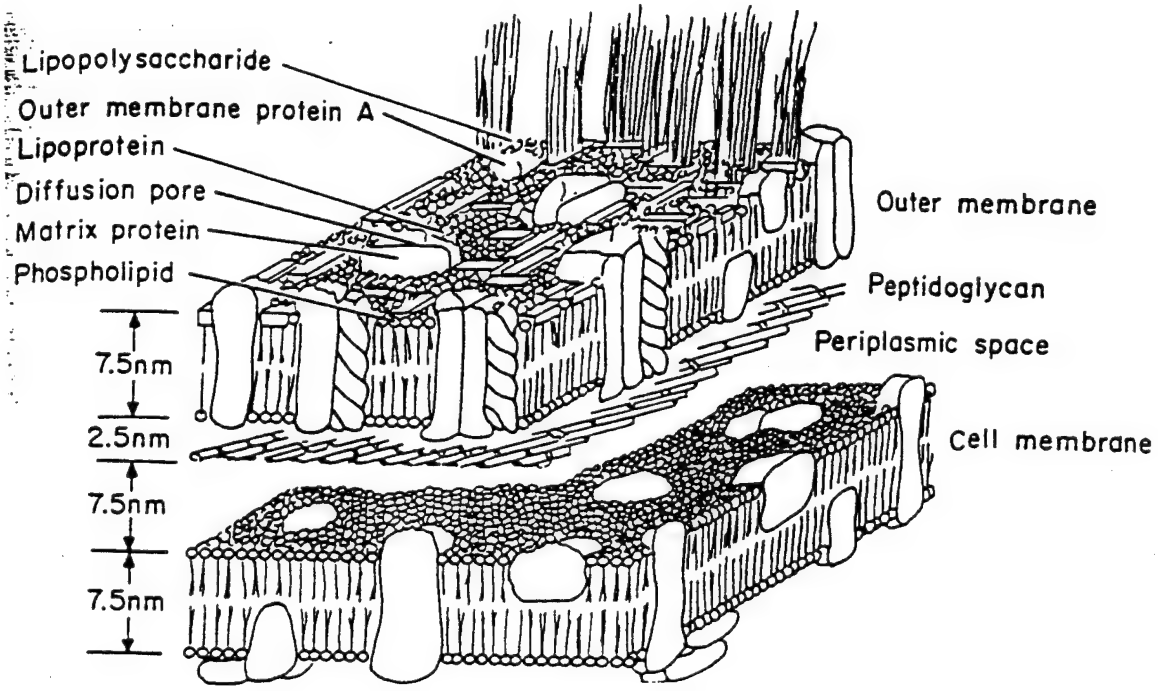


Figure 2-11
Gram Negative Cell Membrane
(Ford and Mitchell, 1992:87)

The cell membrane is made up of an extraordinarily complex combination of proteins and lipids, the ratio of the two and make up can vary greatly with a variety of major and minor polypeptides passing through or studding the membrane (Darnell and others, 1986:570).

In spite of all the differences between Gram negative membranes, a common phospholipid bilayer acts as the main structural unit in all cases (Darnell and others, 1986:570). Phospholipids (Figure 2-12)are amphipathic molecules that contain both a hydrophobic and hydrophilic portion (Darnell and others, 1986:570). The hydrophobic ends will face each other to avoid the polar water surface while the hydrophilic ends will face the polar environment. The polar regions of the phospholipids consist primarily of phosphoryl and carboxyl groups (Flemming and others, 1990:3198) and in general have a net negative charge allowing the walls to interact with cations in the surrounding medium (Beveridge, 1989:152). Some specific structures that have been associated with metal binding include peptidoglycan, terochoic acid, terchuronic acid, lipopolyscharides, and surface protein arrays.

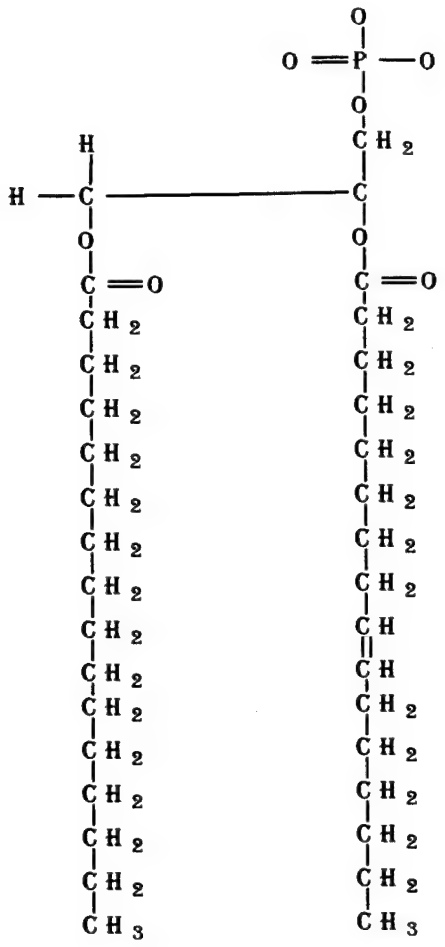


Figure 2-12

Basic Structure of a Phospolipid

(Lehninger, 1982:293)

In addition to the phospholipids, the Gram negative cell membranes also contain proteins. Some membrane proteins are bound to the fatty core of the membrane (Darnell and others, 1986:557). These are called integral membrane proteins or intrinsic proteins and have one or more non-polar regions which interact with the non-polar section of membrane phospholipids. These proteins will most likely but not always contain polar

regions on both sides of the membrane. If a protein spans the entire bi-layer it is referred to as a transmembrane protein (Darnell and others, 1986:578)

The proteins that do not interact directly with the hydrophobic core are called peripheral or extrinsic proteins. They are either bound to the membrane indirectly through interaction with intrinsic proteins or directly through interactions with the polar groups on the surface of the membrane (Darnell and others, 1986:578). (Figure 2-13)

No evidence has been found linking membrane proteins as necessary to maintain the structural integrity of the membrane. Instead data suggests that these proteins are specialized, each fulfilling specific functions (Darnell and others, 1986:572). Bacterial membrane-proteins are most likely used to transport ions, substrates, or growth factors across the cell membrane.

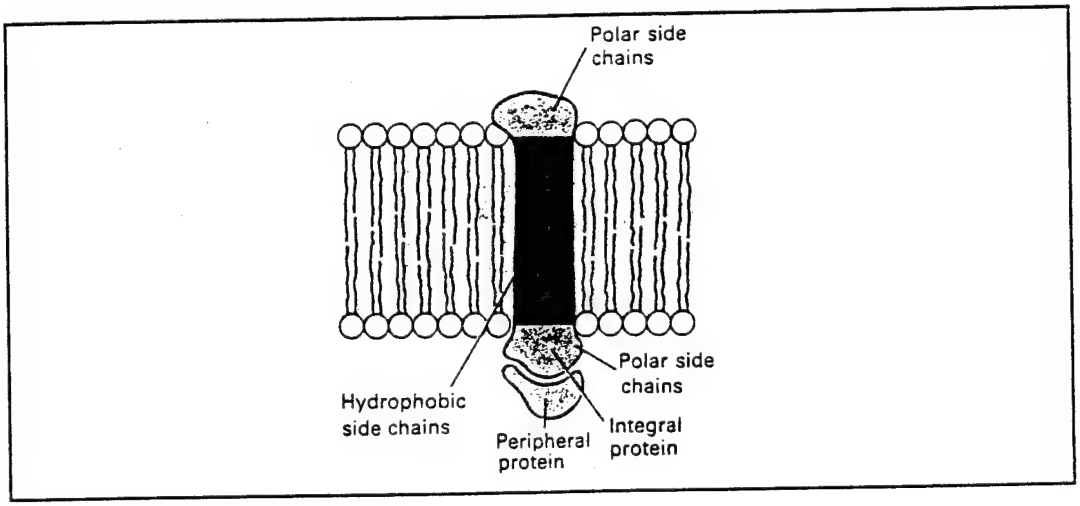


Figure 2-13

Protein Structure of Gram Negative Cell Membranes

2.2.3 Capsules

In addition to the cell membrane many microorganisms possess one or more of the following layers, see Figure 2-11. Of these, the microorganism usually contains a capsule or slime layer. The capsule represents an important component of most bacteria because it facilitates adhesion to surfaces and promotes biofilm formation. It offers protection against antibodies and other adverse environmental conditions (McLean and others, 1990:3675).

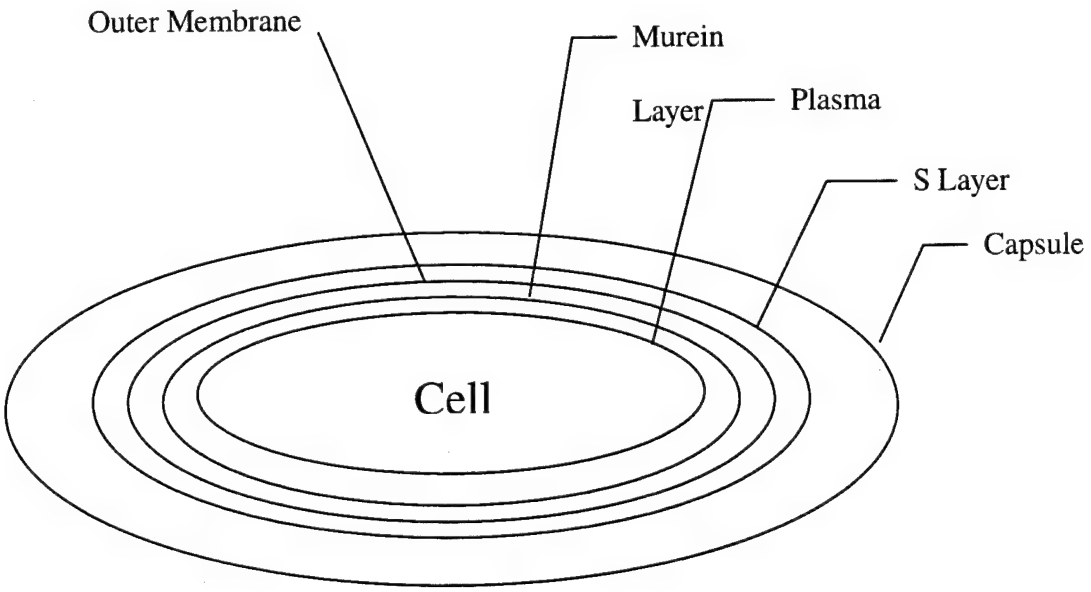


Figure 2-14
Outer Envelope of a Typical Gram Negative Bacteria
(Beveridge, 1989:150)

A bacterium in its natural environment will often possess a cell wall overlain by a multiplicity of superficial layers. However after several sub-cultures in a laboratory medium these layer are no longer required and are lost (Beveridge 1989:148). These

changes in the microbes makes correlation between laboratory studies on metal sorption and mineralization and the natural environment difficult (Beveridge 1989:148). For example, capsulated strains of *Klebsiella aerogenes* were more resistant to Cd than were non-capsulated strains (Babich and Stotzky, 1980:99). This is important in the justification to use fresh mixed cultures in experiments in order to maintain more realistic environmental conditions.

The capsule is a layer that extends 0.5-1.0 micrometers beyond the cell surface. It consists of an amorphous configuration of loosely arranged homopolysaccharides, heteropolysaccharides or polypeptides that are chemically linked (covalently, electrostatically, or by salt bridges) (Beveridge, 1989:154). The capsule is primarily anionic, owing to the presence of carboxylate or phosphate groups (McLean and others, 1990:3671). Its growth is influenced by the quantity of carbon and nitrogen in the growth medium but also by the type and quantity of metallic ions to which the cell is exposed (Beveridge, 1989:157).

The capsule may exist in either a gel or fluid phase with the adsorption of cations and the formation of salt bridges stabilizing the gel phase (McLean and others, 1990:3676). The highly flexible and anionic nature of the capsule make it an excellent bonding site for multivalent metal species. The capsule is better at absorbing metals than the cell membrane because its flexibility (fluidity) is better at adapting to the coordination spheres of a metal. The flexibility of the capsule allow it to surround and enfold a metal adapting to its coordinate sphere (McLean and others, 1990:3676). Capsules will interact with the cations before they reach the cells.

2.2.4 Ionic transport across cell membranes

The protoplasm of the *E. coli* contains approximately 0.3% trace elements. The most abundant of these are manganese, cobalt , copper, zinc, and molybdenum. They are essential for cellular activity even though zinc and copper are toxic in higher concentration (Ford and Mitchell 1992:85). This section will briefly describe the proposed mechanisms for ion transfer across a cell membrane.

In the first type of transfer, lipid permeation, the metal simply permeates the membrane layer. This may work for some ions but the majority can not pass this way (Ford and Mitchell, 1992:84). A second method occurs when ions enter through an ion channel. An ion channel is a hole in the membrane caused by tubular transmembrane proteins (Ford and Mitchell, 1992:84). These channels are small and are used to transport only smaller metal ions.

A third method involves the use of an ion pump. The ion pump transfers ions across a cell membrane and allows them to build up against the ionic gradient (Ford and Mitchell, 1992:84). Still another transport mechanism is complex permeation. In complex permeation the metal complexes with another chelator producing a zero charge molecule. This molecule may now diffuse across the cell membrane into the cytoplasm (Ford and Mitchell, 1992:84).

Another form of ionic transport used to obtain trace elements is carrier mediated transfer. There are two cases of carrier mediated transfers. In the first case, wall attached ligands are thought to bind the ion. The complexed metal is slowly transported into the cell (Ford and Mitchell, 1992:84). In the second case the organism produces and excretes

into the medium an exochelator. (Brady and others, 1994:217). The exochelator binds with the ion in the medium and helps with its mediation across the cell membrane. A specific example of this occurrence is the production of siderphores. Siderphores are organic chelators that are released into the environment to bind iron or other ions (Ford and Mitchell 1992:87). The exochelators may be controlled through feedback mechanisms; an example of this feedback control schematically shown in Figure 2-15 (Ford and Mitchell 1992:86).

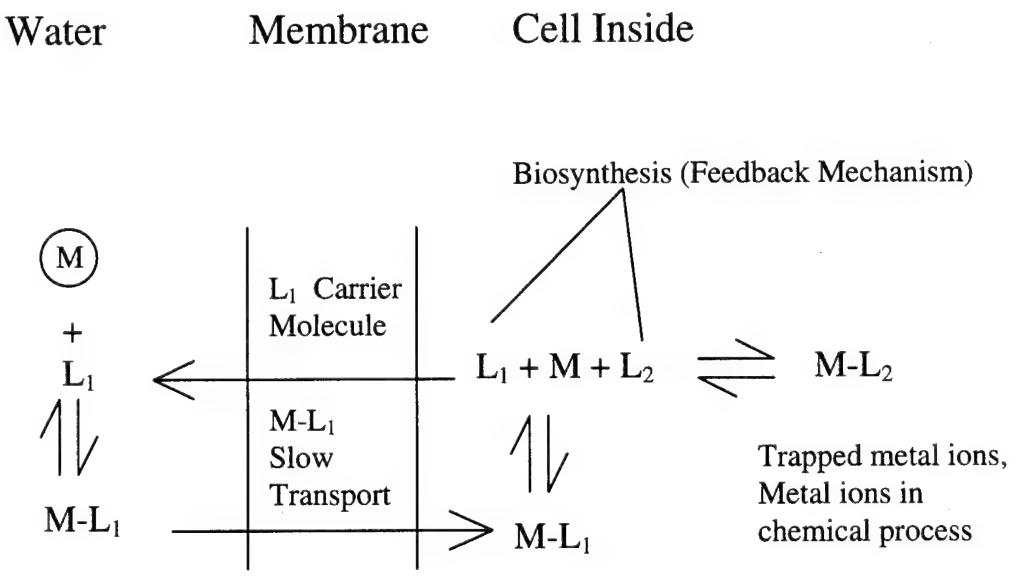


Figure 2-15
Simplified Metal Uptake Model
(Ford and Mitchell, 1992:86)

2.2.4.1 Trace Element Uptake

Uptake of most trace metals is catalyzed by a two step process where the metal is first deposited on the exterior of the cell wall, followed by a slow transfer into the cell.

This process is discussed briefly in section 2.2.4. The steady state transfer of the metals into the cell can be described by the Michaelis-Menton Equations (Morel and Hering, 1993:407).

Reaction: $$M + L \underset{K_{-L}}{\overset{K_L}{\rightleftharpoons}} M \cdot L \xrightarrow{K_{in}} M_{cell}$$

Uptake Rate: $$\rho = \frac{[M]}{K_m + [M]} \cdot \rho_{max}$$

Maximum Uptake Rate: $$\rho_{max} = L_T \cdot K_{in}$$

Half Saturation Constant: $$K_m = \frac{K_{-L} + K_{in}}{K_L}$$

At low concentrations the free metal ion concentration [M] is linearly related to metal uptake. At higher concentrations most of the ligands are already bound to metal ions and thus the uptake rate becomes saturated at ρ_{max} (Morel and Hering, 1993:408).

In many cases the cellular surface may be in competition with other organic and inorganic chelating agents, in which case the metal ligand species dominates the solution. The concentration of metal ion, [M], used in the Michaelis-Menton Equations and representative of the metal that is available for cellular uptake is then a function of the complexation and dissociation constants of the metal and aquatic ligands. This leads to three possible situations: "A) The reactions with the ligands may be fast enough that the trace metal is in a state of equilibrium; B) the rate of binding to the (cell) surface ligand may limit cell uptake; C) the rate of dissociation of the (competitive inorganic/organic) complex may limit metal uptake rates" (Morel and Hering, 1993:408).

Situation A is most likely to occur in this thesis effort. The number of cellular bonding sites is low compared to the inorganic bonding sites and the free metal uptake into the cell is slow compared to all complexing and dissociation reactions. This reaction can be seen schematically in Figure 2-16 where M is the metal of interest, Y is the organic or inorganic ligand in solution competing for the metal, and L is the ligand on the cell surface that binds the metal and mediates its transfer across the cell membrane.

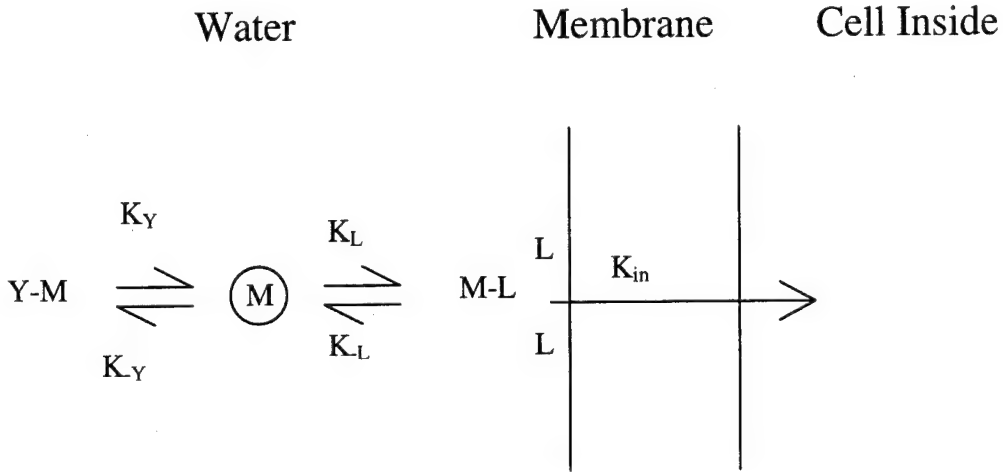


Figure 2-16
Ligand Mediated Metal Transfer Across a Cell Membrane
(Morel and Hering, 1993:409)

This type of cell membrane mediated metal uptake model will be used to determine the importance of free metal concentrations and to model the effect of increasing metal concentration on toxicity, Sections 2.5.5.1 and 2.5.5.2.

2.2.5 Summary

The cellular envelope is made up of a vast complex network of proteins and lipopolysaccharides which are used for structural integrity as well as specific functions.

The envelope is negatively charged and it is possible for cations to bind to it. This binding will be discussed in the following section.

2.3 Metal binding

Cell walls and their various components have a net anionic character and hence will bind with cationic metals. This section will discuss the binding and uptake of metal by the cells. Much of the work concerning metals accumulation has been done on purified cell membranes where only adsorption and surface precipitation are factors. When using whole cells the process of active uptake of the metals into the cytoplasm through non-specific cation transport systems and precipitation of metals at the cell surface must be considered (Mullen and others, 1989:3143). The quantity of metals bound by a cell differs not only between species but also between growth environments (Beveridge, 1989:156). In addition, metal binding capacity is influenced not only by wall chemistry but also by the strength of metal ligand interactions, the size of the metals, and configuration of the ligand (Beveridge, 1989:156).

There are six distinct processes by which microbes interact with cations: intracellular accumulation, cell wall association, metal siderphore interactions, extracellular mobilization/immobilization, extracellular polymer interactions, and transformation/volatilization (Ford and Mitchell, 1992:83). Intracellular accumulation over the life of the organism and cell wall association will presently be discussed; metal siderphore will be discussed in section 2.4.2, Response to heavy metal toxicity. Extracellular mobilization / immobilization, extra cellular polymer interactions and transformation / volatilization will not be dealt with in this paper.

2.3.1 Intracellular Accumulation

The amount of intracellular accumulation is an important key to the cause of a metal's toxicity. If the metal is transported into the cell it may cause toxicity by disruption of cellular functions. If a metal is not transported into the cell the cause of its toxicity must be related to changes in essential cell membrane structure and chemistry. These changes may result in cell lysing, inhibition of carbon source uptake, inhibition of oxygen transport, or the inhibition inorganic nutrient uptake.

Intracellular accumulation is characterized by a fast interaction with the surface ligand followed by a slow transport into the cell. Upon entering the cell, metals are promptly linked to large pools of protein-like substances where they are stored until used for cellular functions (Albergoni and others, 1980:121) . Toxic metals entering the cell will be adsorbed to these substances and may eventually accidentally be incorporated into cellular metabolic activities. Cells may respond to the presence of metals by producing more ligands; it has been shown that high levels of physiological and non-physiological metals can induce the formation of greater amounts of these binding proteins (Albergoni and others, 1980:121).

Research has shown that some metals accumulate on/in the cells rapidly while others accumulate slowly. Vymazal found in *C. colonerota* that metals accumulated at two distinct rates, Ni^{2+}, Cr^{3+}, Fe^{2+}, and Mn^{2+} accumulated steadily over the course of several hours while Cu^{2+}, Pb^{2+}, Cd^{2+}, and Co^{2+} were accumulated rapidly (Brady, and others 1994:213). It is likely that the slow accumulation rate was due to the process of transfer across the cell membrane while the quick accumulation rate was a dominated by

cell membrane adsorption. By determining metal uptake kinetics the distinction between surface adsorption and cellular uptake might be possible.

2.3.2 Cell Wall Interactions

Whether metals are accumulated with in the cell or simply adsorbed to cell membranes, they must interact with the cell membrane. Thus an understanding of metal cellular interactions is necessary. This section will discuss coulombic interactions and precipitation as well as the difference in metal-cell wall interactions between Gram positive and Gram negative bacteria. Cell membrane phosphoryl groups have been suggested as the primary sites for metal interactions (Beveridge, 1989:157) although carboxylate, carboxylic, amino, thio, hydroxo and hydroxycarboxylic groups can interact coordinately with metal ions (Ford and Mitchell, 1992:86). In addition pyruvate, hydroxyl, succinyl and uronic acids may also bind metals (Ford and Mitchell, 1992:90). In fact, Xue et. al. have shown that algal surfaces contain functional groups that bind metals competitively with many dissolved ligands (Xue and others, 1988:917)

2.3.2.1 Coulombic Attraction of Metals and Ligands

Coulombic attraction is one method by which cell membranes might bind metals. These are weak electrostatic attractions that are very non-specific and result in easily exchangeable cation reactions. Most often these result from a substitution of a metal ion for a proton (ion exchange). Below is a model of the substitution reaction as adapted from Flemming et. al. (Flemming and others, 1990:3201)

$$[M^{n+}]+[Cell] \underset{\longleftarrow}{\overset{K}{\longrightarrow}} [M^{n+}cell]$$

$$[H^{+}]+[Cell] \underset{\longleftarrow}{\overset{K_b}{\longrightarrow}} [Hcell]$$

$$[M^{n+}]+[Hcell] \underset{\longleftarrow}{\overset{K-K_b}{\longrightarrow}} [M^{n+}cell]+[H^{+}]$$

Figure 2-17
Model of Coulombic Attraction Between a Metal and Cell Membrane
adapted from Flemming and others, 1990:320

In their work with *Bacillus subtilis* 168 walls and *Escherichia coli* K-12 envelopes, C.A. Flemming and others, were able to adsorb large quantities of Ag^{1+}, Cu^{2+}, and Cr^{3+} to the cell membranes. They attempted to "steal" the metals back through the addition of either nitric acid, Ca^{2+} (competitive cation exchange), or EDTA (chelation of metals). They were able to remobilize large quantities of Cu^{2+}, and Ag^{1+} but were unable to recover a high percentage of Cr^{3+} (Flemming and others, 1990:3194). Darnell, while experimenting with *Chlorella vulgaris,* found that most cations could easily be eluted by reducing the pH to 2; however, Hg^{2+}, Au^{2+}, and Ag^{1+} were firmly bound and could not be released by this method (Darnell, D. and others, 1986:208). It was concluded that the metals that are easily dislodged from a cell membrane are simply undergoing ion exchange while the others are more tightly bound. The more tightly bound ions are usually the result of strong complexation with ligand sites in the cell membrane. (McLean and others, 1994:472)

2.3.2.2 *Precipitation of Metals in Cell Membranes*

Precipitation on a cell membrane is effected by many factors, the type of metal, the pH, and other environmental factors can all have an effect.

Another major mechanism in the cell wall interactions of cations is precipitation (Flemming and others 1990:3198) which is believed to occur in a two step process. First a stoichiometric interaction of the metal with a reactive cell membrane occurs at the bacterial surface (Beveridge, 1989:160). This reaction primarily occurs in the peptidoglycan. (Flemming and others, 1990:3198). The metal ligand complex acts as a nucleation site for the precipitation (Beveridge, 1989:160).

It has been determined that energy dependent mechanisms were not necessary for the accumulation of zinc in *C. glomerate* (fresh water algae) to occur (Vymugal 1987:101). It has also been determined that conformational changes (e.g. salt bridges) may work to reduce the number of potential sites for metal ion binding by rendering some sites inaccessible (McLean and others, 1990:3676). This would lower the saturation point for metal adsorption.

Beveridge and Murray have proposed that in discrete regions of the cell membrane enough bound metal is chemically reduced to provide a nucleation region for the continued accumulation of the metal (Mullen and others, 1989:3149). X-ray analysis by electron spectroscopy (Mullen and others, 1989:3149; Beveridge, 1989:160) has revealed visible precipitation within the cell walls. The nucleation sites, however, are constrained within the interstices of the cell wall and thus only small grain precipitates can occur (Beveridge 1989:160).

R.J.C McLean and others observed a green tint on the cell wall of microorganisms after treatment with copper. This indicates the possible deposition of one or more copper oxides or hydroxide minerals. (McLean and others, 1994:473) Strong Lewis acids such as Mn^{+2} Al^{3+}, Cr^{3+}, and Fe^{3+} can form redox couples which are highly interactive with soluble anions such as OH^- and consequently form insoluble precipitates. These ions can act strongly with biological surfaces and thus nucleate a diverse array of metal aggregates (McLean and others, 1990:3676). The bioaccumulation of these metals has been found to be greatly affected by pH; pH less than 5 and greater than 7 was found to decrease the bioaccumulation of metal (Brady and others, 1994:213). Presumably at lower pH's the hydrogen ion competes with the cation for ligand sites thus at equilibrium less metal is accumulated. At higher pH's, hydroxyl ions compete with ligands to complex the metal ions.

2.3.2.3 Differences Between Gram Positive and Gram Negative

It is widely held that gram positive bacteria generally have a higher capacity for metal sorption than gram negative ones (Beveridge and others, 1989:150). This is primarily due to the thickness and anionic character of the cell membrane primarily resulting from the peptidoglycan, teichoic and teichuronic acids (McLean and others, 1994:472). However, Mullen et. al. in a comparison of metal sorption between *E. coli* and *P. aeruginosa* (Gram negative) and *B. cereus* and *B. subtilis* (Gram positive) bacterium has shown that the Gram negative bacterium tested adsorbed more cadmium than the Gram positive bacterium and that both types adsorbed roughly the same amount of copper. (Mullen and others, 1989:3144) They concluded that it is likely both types of

microbe adsorb metal similarly, but that metals are sloughed off Gram negative walls as soluble wall polymers as wall turnover and autolysis occurs. (Mullen and others, 1989:3147)

2.4 Toxicity of Metals

Many metals are essential for life; however, some metals are toxic if present. Some are toxic if present in large quantities and others are toxic if present in the wrong form. In general, metals can fall into one of four categories with respect to microorganisms. They can be non-essential and non-toxic (aluminum) at higher concentrations, essential and non-toxic (calcium), essential but toxic at higher concentrations (copper) or non-essential and toxic (cadmium) (Ochiai, 1995:479). The essentiality and toxicity are correlated with an element's relative abundance in the environment.

Evolution may have an effect on metal toxicity. Exposure to an abundance of an element during evolution would allow for a greater chance of incorporation into metabolic pathways (Ochiai, 1995:479). In cases where more common elements would not work, trace elements such as Se and Co would become essential. Rare elements or elements that are unavailable to the cell are less likely to become essential. In addition, if a toxic element is not encountered during a microbe's evolutionary development, it is less likely that a natural defense mechanism against the elements toxic effect would be incorporated (Ochiai, 1995:479). If a cell is to adequately cope with heavy metals it must have the ability to selectively uptake these metals in the proper quantity to maintain life.

This selectivity is accomplished in a two step process. The first step is to discriminate in elemental uptake while the second step involves rendering the toxic effects minimal upon entrance into the cell (Ochiai, 1995:479).

The concentration of a metal to which an organism is exposed influences its toxicity. When the concentration of a metal is increased, at first there is no effect. This is because cellular defense mechanisms have not been overcome. At some point these mechanisms will be overcome and inhibition will occur. The adverse conditions can include decreased metabolism, "increased generation times, decreased spore germination, mycelial proliferation, fruit formation and spore production by fungi; decreased nitrogen fixation of blue green algae and lichens; decreased photosynthetic activity of green algae and lichens and inactivation of viral infectivity" (Babich and Stotzky, 1980:104). As metal exposure continues to increase eventually a toxic limit will be obtained. The mechanisms by which the inhibition and toxicity occurs are discussed in the next section.

2.4.1 Mechanisms of Toxicity

There are six proposed mechanisms of toxicity in the cell, most of which stem from the fact that soft acids / class B metals form more stable complex with cellular components than hard acids (Beveridge and Doyle, 1989:37). Metal ions may:

1. block essential function groups of biomolecules such as enzymes,

2. modify the active conformation of biomolecules, especially enzymes. The change in conformational shape may render the molecule non-functional.

3. displace essential metal ions from biomolecules. A toxic metal ion may replace a natural ion in a biomolecule if the affinity for the toxic ion to the ligand site is greater than that of the natural ion, thus resulting in a reduction or lack of activity.

(Bertini and others, 1985:925) Replacement of a hard acid with another hard acid is less likely to have this effect (Beveridge and Doyle, 1989:37).

4. disrupt the integrity of the cellular membrane by toxic ions replacing essential ions in the cell membrane.

5. modify some other biologically active agents

6. bind with biologically essential anion resulting in a decreased level of availability

(Ochiai, 1994:481)

2.4.1.1 Importance of Ion Size in Metal Ion Replacement

If a metal ion is to replace another, the size must be similar to the ion being replaced. In the case of size, the variation in ionic radius amongst common transitions metals is small, ±5% (Ochiai, 1994:480). This further enhances the ability of a toxic metal to replace a naturally occurring element in an enzyme. Some metals exist primarily in an oxyanion form, the variations in size amongst oxyanions is even smaller than the cations typically <5% (Ochiai, 1994:480). Heavy metals are significantly larger than the more common cations thus making substitution more difficult; the exception to the rule is Ca(II) whose ionic radius is similar to that of Cd(II), Hg(II), and Pb(II) (Ochiai, 1994:480).

2.4.1.2 Metal Ion Replacement

Replacement of zinc in essential locations of enzymes will effect enzymatic activity. Bertini and others reported on the activity of zinc enzymes after zinc had been

replaced by another metal. Zinc, because it is stable only in the +2 oxidation state, can not perform in redox reactions. This makes zinc (II) a good catalyst when the role of the metal ion is that of a Lewis acid. Zinc can also stabilize the entire structure of an enzyme. When different metals were substituted for zinc various levels of enzyme activity were observed with zero activity occurring most often. (Bertini and others, 1985:925) Cadmium and mercury have been reported to replace native zinc (II) in many proteins and enzymes (Ochiai, 1994:482). It is suspected that the carcinogencity of Ni(II) is due to its replacement of Zn(II) and or Mg(II) in DNA polymerase (Ochiai, 1994:482). In addition to zinc other native cations have been reported to have been replaced by copper and nickel.

2.4.1.3 Cell Wall Displacement

Metal ions often play an essential role in cell membranes. The flexible nature of cell wall polymers allows them to enfold metallic ions, forming salt bridges (Beveridge, 1989:155). This is necessary in order to assume the correct packing order of phospholipids and lipopolysaccharides of the outer membrane (Beveridge, 1989:157). Calcium and magnesium are usually the preferred metal ions although some metals such as potassium are also found. Experiments using EDTA to chelate metals from gram negative membranes cells showed a dependence on Mg^{2+} for outer membrane integrity (Beveridge, 1989:156) Removal of divalent cations by EDTA treatment could result in the removal of the outer membrane thereby releasing protein-lipopolysaccharides and phospholipid complexes. Removal of the Mg and Ca with EDTA results in cell membranes that were more permeable to a variety of metabolites and more sensitive to

antibiotics. (Beveridge and Doyle, 1989:45) Their replacement by competing metal ions may alter the polymer conformation of the membrane (Beveridge, 1989:155) or seriously alter the wetability of the wall surface (Beveridge, 1989:157).

Substitution of a metallic ion by another is difficult when the metal is bound to a large, somewhat rigid, biomolecule. However, if the protein is flexible, as in the case of cell wall proteins, substitution becomes easier (Ochiai, 1994:482). The acyl chain lipopolysaccharides that inhabit the outer leaflets of the Gram negative cell membrane are more densely packed than their phospholipid counterparts. These regions tend to be anionic in nature and naturally repel each other; it is the stabilizing attraction of divalent cations that allows the tight packing of these molecules. As a result of the cell membrane's dependence on divalent cations, destabilization may occur if an essential ion is substituted. Destabilization may result in membrane blebs soughing off (Beveridge, 1989:161).

2.4.2 Response to Metal Toxicity

There are essentially two responses to the presence of metals, tolerance and metal management. The first is tolerance, this is one of the most important mechanisms for survival. The simplest form of metal tolerance is a system by which metals are detoxified upon entering the cell (Albergoni, and others, 1980:121). All metals seem to be tolerated by organisms to some degree. In metal management, metal ions may be selectively transported into the cell, actively pumped out of the cell, bound in a protective capsule surrounding the cell or chelated by excreted substances to be kept from entering the cell.

2.4.2.1 Metal Tolerance

The degree of tolerance is highly dependent on the organism (Ochiai, 1994:482) When metals enter a cell they are quickly linked to a large pool of substances. The metals are there available to be donated to cellular functions (Albergoni, and others, 1980:121). If grown in the presence of high levels of physiological and nonphysiological metals, the cell will produce a greater amount of these binding proteins. The production of these general ligands is considered a protective mechanism against the toxic effect of the metal ions (Albergoni, and others, 1980:121).

Metal tolerance may be obtained through the use of ionic traps that detoxify a metal upon entering the cell. A nickel tolerant strain of *Cyanobacterium synechoccus* contained large quantities of intracellular cyanophycin granules. These granules strongly bound both Ni and Cu, lessening their toxic effects. (Beveridge and others, 1989:60) A copper tolerant strain of *Scenedesmus* has been reported to produce a metallothionein protein that selectively bound copper when exposed to the metal. *Dunaliella* and *Chlorella pyrenoidsa* produced the same protein when exposed to cadmium (Brady and others, 1994:217).

There is evidence that organisms may be "trained" adapted to tolerate metals. *Botrytis cinerea* and *Chlorella pyrenoidosa* were adapted to tolerate higher concentrations of copper and aluminum respectively (Babich and Stotzky, 1980:100). One method of conferring tolerance is through genetic adaptation. Plasmids are created by metal tolerant microorganisms and transferred to other non-tolerant organisms for

incorporation into its genetic code. *Pseudomonas aeruginosa*, for example, carry plasmids for resistance to inorganic mercury, organomercurials, and hexavalent chromium (Babich and Stotzky, 1980:100). *Staphylococcus aureus* possess plasmids conferring resistance to Cd, Hg, Zn, Pb, and As (Babich and Stotzky, 1980:100).

Metal tolerance is not necessary if the metals can be prevented from entering the cell. The next three sections describe common cellular methods for preventing toxic metals from entering the cell.

2.4.2.2 Selective Metal Uptake

Cells may selectively take up a metal ion. Because the uptake of metals or another substance ultimately depends on its binding to cell membranes or carrier molecules, differences in characteristics between metals may be exploited to preferentially uptake one metal ion over another (Ochiai, 1994:480). For single celled organisms this selectivity is not very great, but it can be improved for multicellular organisms (Ochiai, 1994:481). Cations and oxyanions appear as rather featureless entities to a cell membrane so differences in the following may be used to differentiate one form from another (Ochiai, 1994:480).

a. electrical charge

b. size

c. preferred ligands

d. preferred coordinate structure

In the presence of a large quantity of toxic metals, metal uptake may be reduced by simply lowering the permeability of the cell membrane to all metals (Albergoni and others, 1980:121).

The second mechanism to regulate the internal concentration of a metal and maintain it with in a definite level is the ability to remove, by excretion, metal ions that inadvertently cross the cell membrane.

2.4.2.3 Ion Pumps

In addition to accumulation and selective uptake, metal resistance may be obtained through the use of ionic pumps to remove unwanted metals from the cytoplasm. Cadmium resistance in *Saureus* is brought about by a plasmid and an energy dependent Cd^{2+} efflux system. The system transfers a Cd-H bond for a Cd-Cd bond thus exchanging extracellular H^{+} for intracellular Cd^{2+} (Beveridge and Doyle, 1989:60). Arsenate, arsenite and antimony(II) resistance in *Saurenus* and *E. coli* also occur through energy dependent ion pumps. In the case of arsenate, it is transported from the cell by a phosphate dependent mechanism, whereas arsenite is transported via a phosphate independent system (Beveridge and Doyle, 1989:60).

2.4.2.4 Excreted Exochelators

Metal binding with externally excreted proteins may also play a role in metal tolerance. Copper complexation with hydroxamite and catecholate siderphores in cyanobacteria may play a role in toxicity reduction. The presence of Cu-siderophore complexes may trigger the production of tyrosinase (Ford and Mitchell, 1992:88). In

addition, hydroxamate siderophores may play an important role in the reduction of copper toxicity in cyanobacteria (Ford and Mitchell, 1992:88).

2.4.2.5 Cellular Capsule Binding

In some cases it appears that differences in the binding capacities of a cell membrane and a cellular capsule could act as a selective mechanism. Experiments with the isolated capsule of the *Bacillus licheniformis* determined that more toxic ions were preferentially bound over essential ions. This is contrary to the metal binding preference of the cell membrane. The differences in binding capacity would certainly reduce the binding and ultimately the uptake of toxic metal ions (McLean and others, 1990:3676). This would most likely act as a natural defense to metal toxicity.

2.4.2.6 Metal Binding Capacity of the Cell Membrane

In addition to tolerance and regulation, it has been suggested that the high metal binding capacity of a cell membrane is in its self a type of defense mechanism (Ford and Mitchell, 1992:86) Metals that are chelated to the membrane or precipitated in the cell membrane are no longer available for uptake by the cell. Thus the amount of metal in the experiment is reduced and cellular uptake diminished. Even if the cell membrane blebs the metals will be unavailable for uptake by other organisms (Beveridge and Doyle, 1988:60).

2.4.3 Summary

Metal toxicity can occur by many mechanisms with in a cell and the cell has many defenses against the metals toxicity. The type of metal and the defenses of the cell; however, are not the only factors affecting toxicity; the next section describes environmental factors that may affect toxicity.

2.5 Factors Effecting Toxicity

There are many physiological factors that affect the toxicity of a metal. Factors such as dissolved hydroxy, sulfate, carbonate and other complex species that bind strongly with metal ions are just as important (Beveridge and Doyle, 1989:67). Thus it is not only the total metal concentration that is important, the metal species (Calmano and others, 1992:608) and how well the microorganism competes with aquatic ligands for adsorption of the metal ion that also determine toxicity. It seems that the bioavailability of a metal is what affects its toxicity and not its total concentration. Thus factors that affect bioavailibility also effect toxicity.

The following sections will discuss factors that affect the toxicity of metals; pH, inorganic ligands, organic ligands, competitive cations, and the effect of toxic metal concentrations will also be covered.

2.5.1 Effect of pH

Metal toxicity is affected by pH. In one study, copper toxicity increased with pH while nickel toxicity decreased with pH (Beveridge and Doyle, 1989:37). In another study, nickel showed a decreasing toxicity with increasing pH. Although it could

possibly be surmised that the toxicity was changed as a result of the formation of nickel hydroxide, complexes, this is unlikely. Nickel occurs in the divalent cation state to a pH approximately equal to pH 8.5. Therefore it does not appear to be the formation of hydroxylated species that reduces the toxicity in this case (Beveridge and Doyle, 1989:53).

Cadmium showed an increasing toxicity to *M. lustens*, *Staphylococcus* and *Clostridium perfringens* as the pH was increased from acidic to alkaline levels (Beveridge and Doyle, 1989:53). In cadmium toxicity experiments on *Alcaligenes faecalis*, *Bacillus vermiculatum* and *Aspergillus niger* toxicity increased with increasing pH. It was postulated that the toxicity of Cd may be primarily the result of $CdOH^+$ which may penetrate microbial cells more easily than Cd^{2+}. Other theories for changing metal toxicity with pH include 1) changing pHs have an effect on the physiological state and biological activities of microbes and hence their reaction to toxic substances (Beveridge and Doyle, 1989:53) 2) variation in toxicity may be due to changes in physiochemical parameters that are changed with pH such as hydrolysis and the electric field strength of ligands. (Beveridge and Doyle, 1989:37) The inorganic groups of the cell membranes are affected by pH therefore the affinity of a cell surface for a metal ion will also be affected (Beveridge and Doyle, 1989:53).

The growth of *Chlorella pyrenoidosa* at pH=7 and cadmium levels of 0, 0.25, 0.5, and 1.0 ppm showed a doubling time of 11, 21, 22, and 35 hours respectively when the same experiment was conducted at pH 8 the doubling times were reduced to 11, 16, 17, and 25 hours respectively. The differences in toxicity were correlated with an increase in

the uptake of the metal by the cells. At the 0.5 ppm level cells at pH=7 accumulated twice as much Cd as their counterparts at pH 8. It was postulated that the form of Cd had changed and was effecting cellular uptake (Babich and Stotzky, 1980:110)

Further study has shown that the toxicity of Cd to cells increased drastically as pH increased. *Alcalingens feacalis*, *Bacillus cercus*, *Aspergillus niger*, and *Trichodema verida* all showed increasing toxicity to 10 ppm Cd as the pH was increased from 7 to 8 and finally 9 (Babich and Stotzky, 1980:110).

The toxicity of Cu and Hg to *Fusarium lycopersici* increased with increasing pH. It was postulated that the increase in toxicity was primarily due to the reduced competition for bonding sites between the metal and hydrogen ion (Babich and Stotzky, 1980:111). Inhibition to the growth and photosynthesis of *Chlorella pyrenoidosa* was greatly increased by an increase in pH from 5 to 8. It was again postulated that the increase in toxicity was a result of the decrease in competition for bonding sites.

In addition to toxicity, adsorption to cell membranes may be tied to pH. In experiments with *C. kessleri*, a green alga, zinc and cadmium adsorption was found to be pH dependent. This same experiment also found mercury, chrome and arsenic adsorption to be pH independent (Beveridge and Doyle, 1989:53).

The increase in toxicity and adsorption can be explained by the ligand exchange model presented in section 2.3.2.1; as pH decreases $[H^+]$ increases making hydrogen ions more competitive with the metals for binding spots on the cell membrane.

2.5.2 The effect of inorganic ligands

The presence of inorganic ligands in the water can affect toxicity by altering the bioavailibility of the metal. In studies using *C. vulgaris, H. rivulare, P. lectonema boryanum* and *Anacystis nidulans* increasing the concentration of PO_4^{3-} decreased the toxicity of zinc (Beveridge and Doyle, 1989:56). In addition studies increasing the concentration of CO_3^{2-} and PO_4^{3-} decreased the toxicity of Pb and Ni to fungi (Beveridge and Doyle, 1989:56).

Inorganic anions, OH^- and Cl^-, form coordination complexes creating different speciation complexes. Thus increasing the concentration of an anionic ligand can exert different toxicity to microbes. Babich and Stotzky found that increasing the concentration of Cl^- decreased the toxicity of Cd to *A. niger*, R. *stolonfe*, *Aspergellus conoides* and *aospora*. Zinc, however, was found to be more toxic to coliphages as $ZnCl_3^{1-}$ and $ZnCl_4^{2-}$ than as Zn^{2+} (Beveridge and Doyle, 1989:55). The toxicity of Ni to marine fungi was not affected by the level of Cl^- in solution (Beveridge and Doyle, 1989:55).

Zinc toxicity to mycelial mats of *Trichophyton rubrum* was affected by the metals form; zinc as $ZnCl_2$ or $Zn(NO_3)_2$ exhibited a reduced toxicity over the divalent form of the metal (Babich and Stotzky, 1980:100). A 10^{-2} molar concentration of Cd as $CdCl_2$ had toxic effects on mixed fungus cultures while $CdSO_4$ increased fungal respiration (Babich and Stotzky, 1980:100). In experiments designed to examine the toxicity of vanadium, different inorganic compound exhibited different toxic effects with the sequence of inhibition being Na_3VO_4>$NaVO_3$>$VOSO_4$>V_2O_3 (Babich and Stotzky,

1980:100). The photosynthesis of *Nitzchia delicatissima* was more acutely inhibited by phenylmercuric acetate than an equivalent quantity of diphenyl mercury.

The state of a heavy metal (whether it is in the dissolved aqueous form or insoluble form) greatly effects a metal's toxicity. When a metal exists as a soluble salt it is available for microorganism uptake; however, as a solid the metal is not readily available. For example the addition of 100 ppm, 1000 ppm and 10,000 ppm soluble $CuSO_4$ increased, had no effect, and decreased respectively the ammonification rate of a soil culture while 10,000 ppm insoluble $CuCO_3$ had no effect on the soil's ammonification rate (Babich and Stotzky, 1980:100). In a second experiment 10,000 ppm soluble $ZnSO_4$ reduced soil ammonification while insoluble $ZnCO_3$ had no inhibitory effect (Babich and Stotzky, 1980:100). Experiments with *Aspergillus niger* and lead showed that while free divalent lead inhibited growth, water insoluble compounds of lead (PbO, PbS, $PbCO_3$, and $Pb(OH)_2$ had no toxic effect (Babich and Stotzky, 1980:100).

The effect of inorganic ligands on metal toxicity has shown both increases and decreases in toxicity with increased ligand concentration. Results have consistently shown that metals that precipitate from solution are no longer available to the cell and do not exert toxic influence.

2.5.3 The Effect of Organic Ligands on Toxicity

In addition to inorganic ligands, organic matter present in an ecosystem can influence the mobility and bioavailibility and toxicity of heavy metals. In laboratory

experiments with manmade organic chelators, EDTA reduced the toxicity of Zn to the photosynthesis of *Microcystis aeruginosa.* It also reduced the toxicity of Cu, Zn, Cd, and Pb to *Ditylum brightwellii* and the toxicity of Ni to *K. pneumoniae* (Beveridge and Doyle, 1989:58). EDTA reduced the toxicity of Cu to zoospores of *Phytophthora drechsleri* and increased the survival of *E. coli* and *Phaeodactylum tricornutum* that were also exposed to copper (Babich and Stotzky, 1980:128). The inhibition of growth to *K. pneumoniae* when exposed to Cd or Zn was reduced when the bacteria was also exposed to EDTA. In still another experiment, the addition of soluble humic matter reduced the toxicity of zinc, lead, copper, and mercury to a fresh water photoplankton population (Beveridge and Doyle, 1989:59).

The addition of an organic chelator to an ecosystem causes ligands on the cell surface to compete with the chelator for metal ions (Brady and others, 1994:214). Depending on the metal's attraction to the cellular ligand site, relative to the attraction between the metal and chelator, the adsorption and toxicity of a metal may be greatly affected.

2.5.4 Effect of Inorganic Cations on Metal Toxicity

The presence of other cations in the environment may have an effect on the toxicity of a metal cation. In one case the cations may act to block the sorption of a toxic metal by competing with the toxicant for common sites on the cell surface. If the less toxic metal is successful at dislodging the more toxic metal, a reduction in toxicity will occur (Babich and Stotzky, 1980:106). In another possible mechanism of toxicity

reduction, one metal may adsorb to the amorphous complex of the other metal making it less available to the microorganism (Babich and Stotzky, 1980:107).

Magnesium has been shown to reduce the toxicity of some metals. Increased levels of Mg has been shown to decrease the toxicity of Ni to *B. magsterium*, *B. subtilis*, *E. coli* and *Torula utilis* (Beveridge and Doyle, 1988:56). Magnesium was also successful in decreasing the toxicity of cadmium to *I. reveler* and *Klebseilla pneumonia* (Beveridge, 1988:56).

The inhibition of *Chlorella pyrenoidosa* growth by copper was reduced by the addition of iron (Babich and Stotzky, 1980:106). In addition, the addition of iron was instrumental in reducing the inhibitory effects of nickel on *A. niger* and *Chlorella* sp. (Babich and Stotzky, 1980:106).

Even the addition of toxic cations may sometimes decrease the toxicity of a metal. The growth of *Selanastrum capricornutum* inhibited by copper was increased by the addition of cadmium. For example, cultures of *Selanastrum capricornutum* grown on 10 µg/l Cd and 50 µg/l Cu grew better than cultures grown on 50 µg/l Cu alone (Babich and Stotzky, 1980:106).

Increasing levels of calcium reduced the toxicity of Cd to *A. niger*, of Zn to *H. rivulare*, Zn and Hg to *C. vulgaris*, and Zn to *Achyla* (Beveridge and Doyle, 1989:56). In further experimentation copper and lead adsorption was reduced in *N. muscorum* by the presence of Cu, presumably as a result of the competition between Ca and Cu for cellular bonding sites (Beveridge and Doyle, 1989:56).

In addition to the antagonistic effects of metals, synergistic interactions may also occur. The addition of Cu and Ni had a synergistic toxic effect on the growth of *Selanastrum capricornatum*. *Selanastrum* cultures grown on 0.05 ppm Cu alone had a reduced growth of 22%; cultures grown on 0.05 ppm Ni had a reduced growth of 17%. When the two metals were combined growth was reduced by 82% (Babich and Stotzky, 1980:106). Low levels of Cd when added to Zn had a lethal synergistic effect on *K. pneumonial* but had no more toxicity than an equal quantity of zinc on *S. capricornutum* (Babich and Stotzky, 1980:106).

Research has shown that the addition of competitive cations may have the effect of either decreasing or increasing toxicity depending on the metal's combination and the type of microorganism.

2.5.5 Metal Concentration Effect on Toxicity

The concentration of a metal can have a large effect on toxicity. At low levels metals which are normally toxic may stimulate metabolic activity. This may reflect an Arndt-Schulz effect "where the accumulation of a non-lethal concentration of a poison at the surface of a cell induces an alteration in the permeability that permits a freer flow of nutrients across the plasma membrane and there by an increases in cellular metabolic activity" (Babich and Stotzky, 1980:105). Nitrogen fixation in *Nostoc sp* was stimulated by concentrations of Cd, Pb, and Zn between .005 and .025 ppm; however, with metal concentrations between .025 and .125 ppm nitrogen fixation was inhibited (Babich and Stotzky, 1980:105). Exposure of *Lactobaciellus acidophilus* and *Streptococcus facecales*

to 40 ppm cadmium inhibited growth; however, exposure to 20 ppm and 10 ppm respectively stimulated microorganism growth.

Once a metal tolerance limit has been exceeded, additional metals will cause increasing toxicity to the microorganisms. In the next two sections the importance of free metal ion concentrations and the effect of total metal concentrations on toxicity will be investigated.

2.5.5.1 *Importance of Total Metal Concentrations*

The effect of increasing metal concentration may be modeled. This section relies on the model presented in section 2.2.4.1. In this section it was shown that the concentration of free metal in the aquatic environment was an important factor in the determination of the uptake kinetics of metals (both essential and toxic) as the free metal concentration increased $\rho \rightarrow \rho_{max}$. This meant maximum growth in the case of essential metals and maximum inhibition in the case of toxic metals was approached and free metal concentrations are increased.

In our experiments the total and not free metal ion concentration is controlled, therefore a relationship between free metal and total metal must be made in order to relate total metal to toxicity. Although the total metal and total ligand concentrations are varied, the pH, major ion concentrations, and dominant ligand concentrations are kept constant. This means that the free metal concentration is proportional to the total metal concentration (Morel and Hering, 1993:410).

2.5.6 Summary

Research has shown that metals are very reactive with elements in the environment. The alteration of these factors may have a significant effect on toxicity and metal adsorption by the cell.

2.6 Literature Review Summary

This literature review has examined the general physiology of the cell membrane and cellular metabolism and suggested mechanisms of toxicity for metals. It has discussed the toxicity of metals to microorganisms and the factors which affect the toxicity. Chapter three of this paper will discuss the methodology used in this research to determine what the toxic effect of metals is on a toluene selected bacterial culture and what affect environmental changing environmental factors have on that toxicity. Chapter four will provide the results of the experimentation and will attempt to explain the observed phenomena.

III. Methodology

This chapter will discuss the methodology used in this thesis effort. It will discuss the culturing of microorganisms used in this project, as well as the preparation of all standards. It will layout all standard handling procedures used in the experiment as well as describe the experiments performed in detail. In addition, this chapter will discuss x-ray spectrometry and the statistics used in the analysis of results.

3.1 Microorganisms

In order to support the large number of experiments conducted in this thesis, a large source of microorganisms was needed. The culture of microorganisms used must have a fairly stable population size and species diversity to provide reproducible results. This section will describe the method of obtaining the microorganisms and how they were grown.

3.1.1 Microorganism Source

The microorganisms used in this experiment were obtained from the outlet of Tank 4 of the Fairborn water reclamation plant and represent the last stage of the aerobic reaction chamber before the clarifier tank. One liter of the activated sludge was removed and placed into a plastic container for transport to the laboratory.

3.1.2 Microorganism Innoculum

In this step two separate cultures were created from the activated sludge and eventually combined in a bioreactor (Section 3.1.3). The microorganisms were selected

for their ability to degrade toluene. The activated sludge obtained from the Fairborn water reclamation plant was allowed to settle and 40 ml of the light fluffy flocculate was taken from the surface of the particulate layer and placed into a one liter flask. 600 ml of growth solution (see section 3.2.1) was added to each flask and a test tube of toluene was suspended above the fluid on a wire. The toluene evaporated into the air and dissolved into the water. The water was constantly stirred with a magnetic stirrer, creating a one to two inch vortex to entrain more oxygen and toluene. A piece of aluminum foil served as a cap restricting but not eliminating the exchange of gasses with the environment. This provided an environment for the culture to begin to grow. After two days of lag period a toluene selected population began to emerge. The toluene selected cultures were transferred to the bioreactor.

3.1.3 Bioreactor

The bioreactor provided a very large source of innoculum, assuring that each experiment run on a particular day would have a similar population size and make-up. At the end of each day the quantity of innoculum consumed in experimentation was replaced.

The growing culture must receive a constant supply of oxygen and carbon (toluene); the bioreactor received both from variable volume air pumps which forced toluene saturated air through a porous aeration stone. Because toluene was lost from the bioreactor as additional air was passed through the porous stone, the reactor was placed under a fume hood to prevent toluene fumes from accumulating and becoming an explosion hazard.

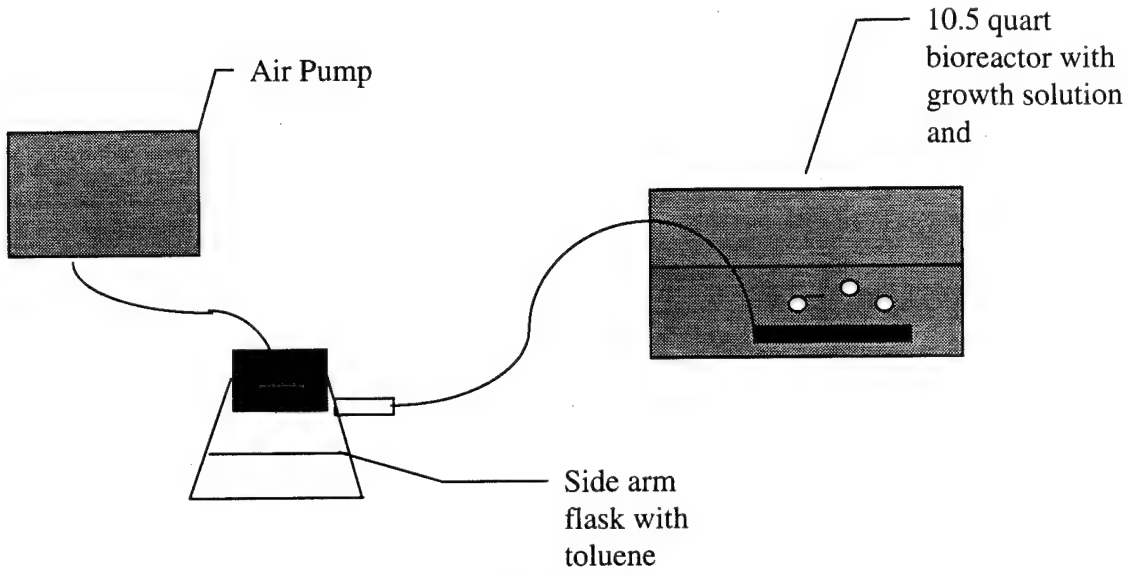


Figure 3-1
Schematic of the Bioreactor

3.2 Growth / Dilution / Stock Solutions

This section describes the preparation of all standard solutions used in this research effort.

3.2.1 Growth Solution

The microorganisms were grown on an inorganic growth solution consisting of distilled water and Stern's Miracle-Gro®. The growth solution for the entire thesis was mixed all at one time; ten liters of distilled water was mixed with 6.666 ±.005 g. of Miracle-Gro, and two six-liter Hach BOD buffer pillows. This solution was then diluted one to one with distilled water to replace the consumed innoculum in the bioreactor. The growth solution consisted of:

Table 3-1

Innoculum Growth Medium

Nutrient	Concentration ppm	Nutrient	Concentration ppm
N	46.02	B	.06
P	90	Cu	.21
K	83	Fe	.6
Mn	.15	Zn	.14
Mg	13.5	Ca	16.5
Cl	40		

When compared to other growth solutions this is quite sparse. However, as previously stated, the toxicity of metal ions is affected by the presence of other metals and ligands; Miracle-Gro represented a large uncertainty because it is a commercial grade product and not reagent grade product. By minimizing the quantity of this unknown, uncertainty in the toxicity experiments will be limited.

Miracle-Gro was selected as the source of nutrients for the growth solution because government supply could not procure in a timely way the necessary essential growth elements to mix a growth solution. Eventually we were able to obtain ammonium dihydrogen phosphate, ammonium nitrate, and potassium nitrate to use in the dilution water, the generous gift of Dr. Audrey McGowin at Wright State University. Most of the other supply items used in the thesis were purchased by the student or professor. We did not obtain the essential nutrients to replace the Miracle-Gro until well into the thesis effort. The Miracle-Gro was working well and its concentration was small compared to

other controlled elements in the experiments so the decision was made to continue using it.

3.2.2 Dilution Water

Dilution water is that water which is added to the innoculum of microorganisms to provide a suitable living environment for the microorganisms during experimentation. It must be homogeneous between experiments in order to minimize variation in the experiment due to differing pHs, and nutrient levels. In an attempt to achieve homogeneity a concentrated solution of nutrients was made up consisting of 5 g/L of the following chemicals: ammonium dihydrogen phosphate, ammonium nitrate, and potassium nitrate. The nutrients were weighed in tarred boats to ± .005 g and dissolved in distilled water whose amount was also determined by weight to ± 2.5 g. The pH of the concentrated dilution water was then adjusted to a pH of 7.0 ± .1 with the addition of concentrated potassium hydroxide.

Table 3-2

Concentration of Nutrients in Dilution Water

Nutrient	Concentration (ppm)
NH_4^+	122
NO_3^-	369
$H_2PO_4^-$	234
K^+	107

3.2.3 Concentrated Stock Metal Solutions

Metal stock solutions were made up at metal concentration of 10,000 PPM in 100 or 200 ml flasks. The mass of the metal salt necessary was determined by taking the ratio of the molecular weight of the metal to that that of the formula weight of the salt. That mass of metal salt was then added to the volumetric flask. The following is an example calculation for copper when the salt added was $Cu(CH_3COO)_2*H_20$. Table 3-3 summarizes standard solutions used in this experiment.

Element	Mol Wt (# of atoms in the molecule)
Cu	63.54
C	12.011 (4)
H	1.00 (8)
O	16.00 (5)
Formula Wt.	199.65

$$x = 10{,}000\frac{mg}{L} \cdot .1L \cdot \frac{199.65\frac{gm}{mol}}{63.546\frac{g}{mol}} \cdot \frac{1g}{1000mg} \qquad (3.1)$$

$x = 3.1418g$ This is the number of grams added to 100 ml to form a 10,000 ppm stock solution.

Table 3-3

Stock Metals Concentrations Used in the Experiments

Metal	salt form	molecular weight (g/mol)	formula weight (g/mol)	Theor weight (g)	actual weight (g)	actual conc. PPM
Cu	$Cu(CH_3COO)_2*H_2O$	63.54	199.65	3.141	3.1418	9999
Mn	$MnCl_2*9H_2O$	54.9	197.91	3.6024	3.6048	10,000
Zn	$Zn(NO_3)_2*6H_2O$	65.39	297.47	4.549	4.5485	9999
Fe	$Zn(NO_3)_2*9H_2O$	55.9	404.00	7.23	7.2383	10,015
Co	$Zn(NO_3)_2*6H_2O$	58.93	290.95	4.937	4.9405	10,006
Mg	$Zn(NO_3)_2*6H_2O$	24.30	256.03	21.307	21.3074	10,111
Ca	$Zn(NO_3)_2*4H_2O$	40.08	236.08	11.780	11.785	10,003

3.3 Microcosm Preparation

The following section describes the standard method used to prepare the microcosms used in this thesis effort. Figure 3-2 gives a flow chart of the entire process, combining the standard methods of the entire section.

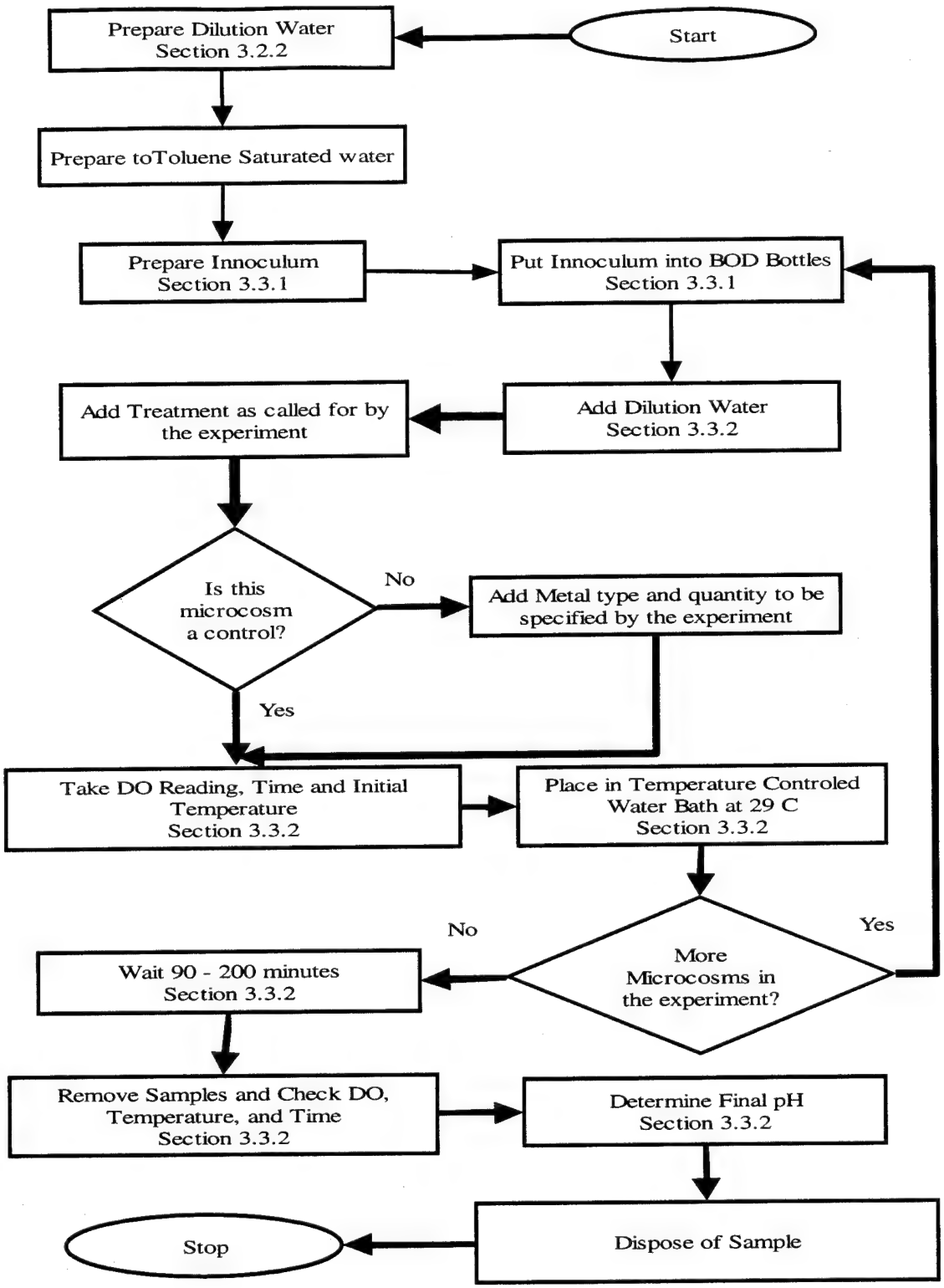


Figure 3-2

Flow Chart of the Steps to Prepare a Microcosm

3.3.1 Aliquots of Microorganisms

Scoping experiments demonstrated that the logistics of one individual preparing microcosms, measuring dissolved oxygen (DO), and handling BOD bottles would limit work to only six samples at a time. This allowed for a control and five treatments in some experiments and triplicates of a control and a treatment in others. Through trial and error a reproducible procedure was developed to place homogeneous aliquots of microorganisms into each of the six BOD bottles.

The steps in the procedure are described in the following paragraphs. First the innoculum was ultrasonicated for three minutes; this aided in breaking up floc and ensuring an equal quantity of microorganisms were placed in each sample. Experiments determining the respiration rate of ultrasonicated and non-ultrasonicated samples showed virtually no difference in respiration rate, indicating that little or no damage was done to the microorganism population due to sonification.

Next three individual 10 ml samples of microorganisms were placed into six BOD bottles; the order of sample preparation which gave the most reproducible results is described in figure 3-3 below where the numbers represent the order in which 10 ml aliquots were deposited into the sample bottles.

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	2	3	4	5	6
12	11	10	9	8	7
18	16	14	13	15	17

Figure 3-3

Order of 10 ml Samples of Innoculum Addition to a Six Sample Experiment

3.3.2 Handling of Microcosms

After the innoculum of microorganisms was placed into the BOD bottles and dilution water added, the microcosm was then treated with the metal(s) in accordance with the experiment. The microcosms were handled in a standardized manner, as described in the following paragraphs.

The DO for each sample was then taken three times, the time and starting temperature were also noted. The toluene equilibrated water was added bringing the toluene concentration in the microcosm to 26 PPM. The BOD bottle was then capped and set in a water bath at 29 ±1 °C for 90 to 200 minutes, depending on the metabolic activity rate of the innoculum. The bottles were removed and the final DO, time, and temperature measured.

Because no attempt was made to maintain a constant pH, the pH of each microcosm has to be tested before disposal. The pH check was to determine if the Lewis acid nature of the metal had significantly changed the pH of the microcosm. If the metal was a particularly strong Lewis acid, the drop in pH caused by the metals complexation with hydroxide might account for increased treatment toxicity beyond the metals toxicity. This was found to be the case only in iron.

3.3.3 Reproducibility

In this thesis the assumption is made that the treated and untreated samples are identical in every way except for the metal treatment. This was accomplished through the use of standardized microcosm preparation. The reproducibility of this procedure was tested in two preliminary experiements, Table 3-4 A and B. In two cases four identical treatments were prepared. The difference between the highest and lowest was about 5% in one trial and about 2% in the other.

Not only is it essential that treated and untreated samples be identical, but it is also necessary that the samples in replicate experiments be identical. This is virtually impossible because we are dealing with a microbial community. The types of organisms and their relative numbers may change with time. In addition, the size and activity of the microbial population may change with time. In an attempt to minimize this variability all microorganisms for a particular trial of an experiment were removed from the bioreactor at one time. The innoculum of microorganisms for each experiment were drawn from this sample while it was continuously stirred by a magnetic stirrer. All dilution water, nutrients, and hydrocarbons came from a common source.

Table 3-4a

Reproducibility of Technique as Demonstrated in Trial Number 1

	Sample 1	Sample 2	Sample 3	Sample 4
DO initial (mg/L)	6.64	6.94	6.86	6.97
Time initial	15:36	15:38	15:40	15:42
DO final (mg/L)	4.75	5.11	4.93	5.12
Time final	19:00	19:02	19:04	19:06
Respiration Rate (mg/L/min)	.0225	.02178	.0229	.0220

Table 3-4b

Reproducibility of Technique as Demonstrated in Trial Number 2

	Sample 1	Sample 2	Sample 3	Sample 4
DO initial (mg/L)	6.51	6.62	6.71	6.69
Time initial	14:56	14:59	15:01	15:03
DO final (mg/L)	4.41	4.51	4.64	4.60
Time final	17:09	17:11	17:12	17:14
Respiration Rate (Mg/L/min)	.0158	.0160	.0158	.0160

3.4 Respiration Rate

The purpose of this thesis was to establish the toxic effects of metals on microorganisms. The respiration rate of oxygen was determined to be a convient means of determining metabolic activity therefore we will measure toxicity as the inhibition of metabolic activity. The following section describes the calculation of respiration rates and how they can be used to determine toxicity.

3.4.1 Determining Respiration Rate

The consumption of toluene and the metabolism of microorganisms is not measured directly in this thesis. Metabolism is measured indirectly by the monitoring of the consumption of oxygen. The general form for aerobic metabolism is :

$$C_m H_n + \frac{3}{2} O_2 \rightarrow H_2O + CO_2 + C_{m-1} - H_{n-2}$$

and for toluene

$$C_7H_8 + \frac{18}{2}O_2 \rightarrow 4H_2O + 7CO_2$$

The rate of oxygen consumption is therefore proportional to the rate of microorganism metabolism. It has been established that the study of oxygen consumption is a good indicator of metabolism (Naziruddin, and others, 1995:151; Grady and others, 1989:957). Oxygen consumption curves have been successfully to model organism growth and even determine Monod growth parameters.

Scoping experiments were conducted in which the dissolved oxygen content of a sample of toluene-selected organisms was continuously monitored. The dissolved oxygen consumption increases linearly over time (Figure 3-4). This is indicative of a very slowly growing population of microorganisms or a population of microorganisms whose size is very large compared to its reproduction rate.

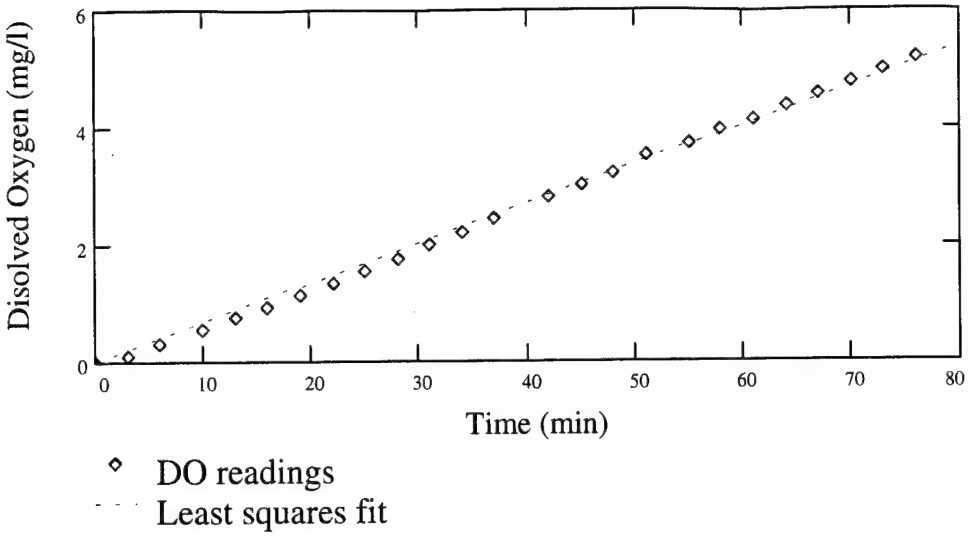


Figure 3-4

Oxygen Consumption as a Function of Time for a Given Aliquot of Microorganisms

mg of Oxygen consumed by microorganisms are plotted against time to show that oxygen consumption is linear.

Because we are dealing with a slow growing population of organisms it was decided that a first order rate of oxygen consumption can be used to describe the metabolic activity of the selected culture. This rate would be determined in most cases by taking three initial dissolved oxygen concentrations and three final concentrations and dividing by the elapsed time between measurements.

$$Rate = \frac{DO_{initial} - DO_{final}}{\Delta Time} \tag{3.2}$$

3.4.2 Dissolved Oxygen Meter (YSI Incorporated, 3-7)

A dissolved oxygen meter was used in the experiments to determine the DO consumption. The dissolved oxygen meter used was the Yellow Springs Instruments

Model 58 dissolved oxygen meter with YSI 5720A self stirring BOD bottle probe. The probe is accurate to ±.03 mg/l in the 20.00 mg/L dissolved oxygen mode.

The BOD probe works using a principle of electroanalytic chemistry. The anode of the sensor is silver while the cathode is gold. The two elements represent the two half cells in a oxygen reduction reaction. The anode and cathode are immersed in an half-saturated solution of KCl with Kodak photo flow and are separated from the environment by a .001 inch FEP Teflon membrane. The membrane allows oxygen and certain other gases to enter. If a polarizing voltage is placed across the poles oxygen that has passed through the membrane reacts with the cathode to produce a current.

The membrane passes oxygen at a rate proportional to the partial pressure in the medium where oxygen is rapidly consumed by the probe. The partial pressure of oxygen in the probe is essentially zero. Therefore the rate of oxygen flow across the membrane is directly proportional to the partial pressure of oxygen in the solution. If the pressure of oxygen decreases in the environment the amount of oxygen passing through the membrane will decrease, producing less current. The current produced by the probe is amplified by a high impedance amplifier and reported in terms of mg O_2/l. It is these readings that are recorded and used to compute metabolic activity rates and inhibition.

3.4.3 Metabolic Inhibition

The toxicity will be measure as metabolic inhibition. In this case the toxic effect of a metal treatment will be determined by the ratio of the rate of oxygen consumption of a treated sample divided by the rate of oxygen consumption of an identical untreated

sample. This ratio will be termed the normalized metabolic activity (NMA) rate. A NMA of 1 indicates that there is no metabolic inhibition by the metal treatment, while an NMA of 0 indicates complete inhibition / toxicity by the metal treatment.

$$NMA = \frac{\frac{DO_{initial(w/metal)} - DO_{final(w/metal)}}{\Delta Time_{(w/metal)}}}{\frac{DO_{initial(control)} - DO_{final(control)}}{\Delta Time_{(control)}}} \qquad (3.3)$$

The ratio of oxygen consumption rates is used to compensate for differences in microbial population size and activity between experiments. This will allow for comparison of the effects even though the initial populations may be very different.

To show that the normalization of a treated metabolic rate to an untreated metabolic rate will account for differences in microbial population size and activity scoping experiments were conducted. In scoping experiments the quantity of innoculum was varied and the rate of oxygen consumption determined (Figure 3-5 A and B). The rates were then fitted with a linear regression line. As can be seen, the rate of oxygen consumption varies linearly with the quantity of microorganisms.

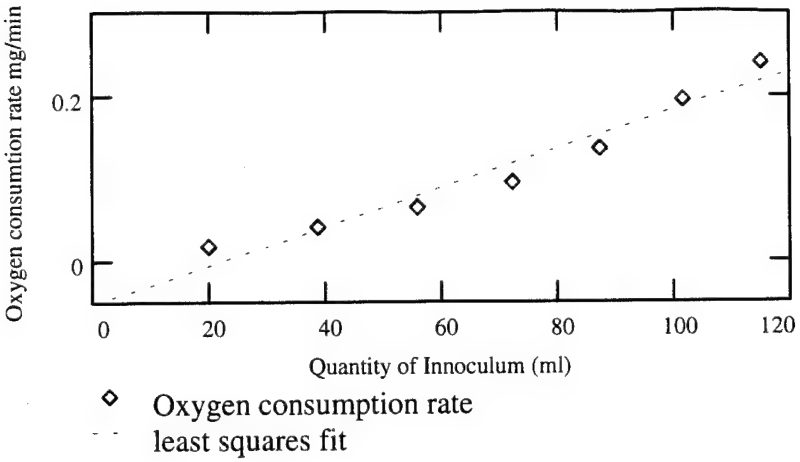


Figure 3-5A Trial #1

Relationship Between Microbial Population Size and Respiration Rate

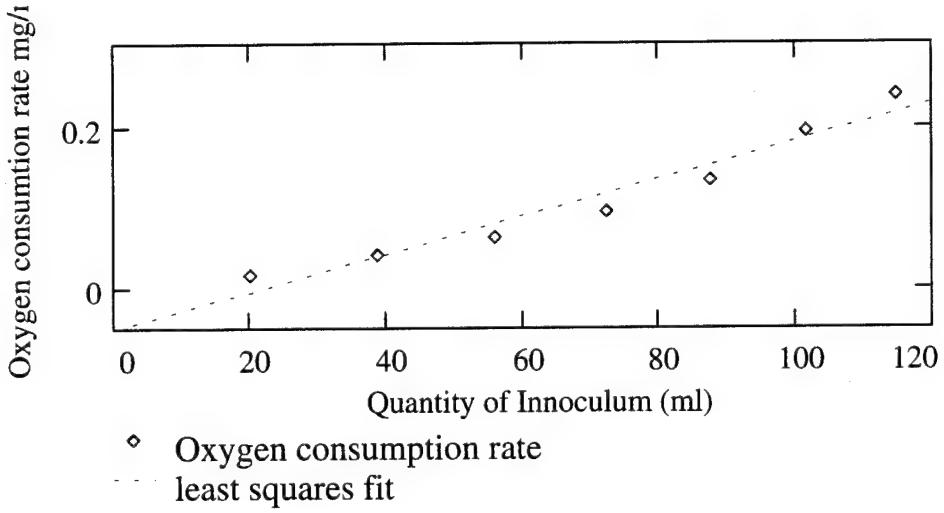


Figure 3-5B Trial #2

Relationship Between Microbial Population Size and Respiration Rate

3.5 Metabolic Inhibition Experiments

Inhibition will be interpreted in this thesis from the normalized metabolic activity rate (NMA). This will be used as the dependent variable in all toxicity experiments. An NMA of one will indicate that there is no inhibition caused by the metal treatment while an NMA of zero indicates that all metabolic activity in the treated sample has stopped. The following section describes the experiments performed in this thesis to test for metal inhibition and the factors that affect inhibition.

3.5.1 Metabolic Inhibition as a Function of Metal Concentration

The purpose of this experiment was to determine the response of a microorganism culture to varying concentrations of a particular metal. In this experiment 30 ml aliquots of microorganisms were placed in 300 ml BOD bottles as described in Section 3.3.1. Dilution water prepared as described in Section 3.2.2 was added. A varying quantity of the metal of interest was added. The sample was then handled as described in Section 3.3.2.

In all cases the metal was tested at 0, 4, 8, 16, 33, and 50 ppm. For copper, which was the most toxic metal tested, toxicity experiments were run at 0, 1, 2, 3, 4, and 5 ppm. While for Mn, which was relatively non-toxic, additional experiments were run at 0, 50, 100, 150, 200, and 250 ppm. The results of the 0 ppm metal sample were used as the control. The control rate was used to normalize the metabolic inhibition of the metal. The experiments were repeated 3 times for each metal.

The results were then plotted as NMA vs. total metal concentration for each replicate and each metal. No statistical analysis was performed on these experiments, their purpose was to understand the trend in toxicity of increasing the metal concentration.

3.5.2 Inhibition as a Function of pH

In this experiment the effect of pH on metal toxicity was investigated while at the same time the relative metabolic inhibition of each metal was compared to the others. This was accomplished using a two factor design; the experimental matrix can be seen in Figure 3-6. Each metal was tested at a single concentration at three pHs 5, 7, and 9. Six treatments were prepared in each experiment. One sample contained no metal and was used as the control. One sample was used for each of the 5 metal treatments. The innoculum was prepared as described in Section 3.3.1 and 30 ml was placed in each BOD bottle. Dilution water, as described in Section 3.2.2 was added; the microcosm was then handled as described in Section 3.3.2.

The respiration rate for the control sample was used with the treatment respiration rate of each metal. Three experiments were run with each batch of innoculum. Metal toxicities were determined for all metals at each pH level as described in Section 3.4. Making the assumption that the population size and health was the same for each experiment allows us to compare metal toxicities across pHs.

	Control	Cu	Co	Fe	Mn	Zn
pH=5						
pH=7						
pH=9						

Figure 3-6

Matrix Design of pH Effects on Metal Inhibition

When the three treatment level were investigated and the experiment complete, a new batch of innoculum was obtained from the bioreactor and the experiment was repeated.

3.5.3 Effects of Magnesium and Calcium on inhibition

The purpose of these experiments was to determine if calcium or magnesium ions are capable of reducing the metabolic inhibition of copper, cobalt, or zinc.

These experiments were very similar to the pH experiments in design; three treatment levels were chosen: 0 ppm, 50 ppm and 100 ppm Ca or Mg added. The experimental design matrix is shown in Figure 3-7. Six samples were used in each treatment; three samples were controls and received a 30 ml aliquot of microorganisms as described in Section 3.3.1 and dilution water at pH=7 produced as described in Section 3.2.2. The appropriate amount of Mg or Ca was placed in each control. Three sample bottles were prepared in the same fashion as the controls, but were treated with eight ppm of one of the metals tested. The samples were then handled as described in Section 3.3.2. Two replicates of the experiment were completed with new batches of innoculum.

	Cu	Co	Zn
0 PPM Mg or Ca			
50 PPM Mg or Ca			
100 PPM Mg or Ca			

Figure 3-7

Experimental Matrix Design of the Effect of Mg^{2+} or Ca^{2+} Cations on Metabolic Inhibition

3.5.4 Sterile Plating Technique

Sterile plating techniques were used in order to get a measure of the size and diversity of our toluene-fed culture as well as to get an idea of the lethality of the metals being tested.

The laboratory work area was first wiped with alcohol to sterilize it. Next all the equipment was laid out. Sterile disposable pipettes were employed to prevent cross contamination between cultures, sterile Millipore filters and clean Millipore filter holders were also employed. All sterile handling was accomplished with tweezers that had been sterilized in the flame of an alcohol lamp.

For this experiment 300 ml of innoculum were removed from the bioreactor and ultrasonicated for three minutes to break up any flocculation that might have occurred. 20 ml of innoculum were placed in new scintillation bottles and dosed with 1 ml of 10,000 ppm metal stock solution. This is the equivalent of the 50 ppm that the 30 ml of innoculum would have seen in the experiments.

After a 15 minute contact period 1 ml of metal dosed microorganisms was removed and placed into 99 ml of sterile Hach dilution water. This is effectively a 1:100 dilution of microorganisms.

Petri dishes were next prepared. Each metal was tested on three media, Hach M-Endo a medium designed for the growth of *E. coli* bacteria. Hach M-TCG a general heterotrophic medium, and M-Green a medium designed for mold and fungi growth. The ampoules were first swabbed in alcohol to sterilize them and then broken and the contents poured onto the petri dish pads.

A sterile Millipore filter was placed on the filtering apparatus and 1 ml of the diluted microorganisms was passed through the filter. The filter was removed and placed atop the nutrient medium. The petri dish was then incubated at 34 °C for 24 hours. The filtration technique was repeated for each growth medium and each metal.

After 24 hours the petri dishes were removed from the incubator and the number of colonies and the number of colony types were counted with a 4 power magnifying glass. In the case of the M-TCG the colonies were too numerous to count (TNTC). Estimates were made by counting the number of colonies in 5 randomly selected grids and averaging the number of colonies per grid. The number of affected grids were then determined and the number of organisms estimated. The different type of colonies were determined by looking at the differences in colony shape, color and size. No attempt was made to classify these microorganisms or to count their relative numbers on the petri dish.

3.5.5 *Effect of Population Size on Metabolic Inhibition*

In this experiment the effect of the population size on metal toxicity was explored for copper, cobalt, and zinc using a two factor experimental design matrix (Figure 3-8).

	Cu (16 PPM)	Co (33 PPM)	Zn (33 PPM)
10 ml innoculum			
30 ml innoculum			
50 ml innoculum			

Figure 3-8

Experimental Design of the Effect of Microbial Population Size on the Toxicity of Metals

Three treatment levels of microorganisms were investigated, the organisms were tested at 10, 30, and 50 ml of innoculum for each of the three metals. Six samples were made for each level of innoculum. Three were controls while three were replicates of the treated sample.

The microorganisms, prepared as described in Section 3.3.1, were pipette into 300 ml BOD bottles; for the 10 ml innoculum and 50 ml innoculum treatment levels a modified procedure was used. For the 10 ml treatment only the first six steps of the procedure described in Section 3.3.1 were used (Figure 3-9). For the 50 ml innoculum the entire procedure as described in Section 3.3.1 was completed and the first 12 steps of the procedure was repeated (Figure 3-10). The 30 ml treatment was prepared as described above.

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	2	3	4	5	6

Figure 3-9

Order of 10 ml Samples of Innoculum Addition to a Six Sample Experiment

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	2	3	4	5	6
12	11	10	9	8	7
18	16	14	13	15	17
19	20	21	22	23	24
30	29	28	27	26	25

Figure 3-10

Order of 10 ml Samples of Innoculum Addition to a Six Sample Experiment

Next dilution water, prepared as described in Section 3.2.2, was placed in the BOD bottle along with the appropriate metal concentration. The microcosms were each handled as described in Section 3.3.2.

A single sample of microorganisms was removed from the bioreactor for each experiment. The sample was prepared as described above and was used for each treatment level for a single metal. This helped to provide homogeneity between treatment levels for each metal.

3.5.6 The Effect of Inorganic Nutrients on Metabolic Inhibition

This experiment explores the changes in metabolic inhibition for copper, cobalt, and zinc for three treatments of inorganic nutrients. It is an attempt to determine if nutrient levels can affect the metabolic inhibition of a metal.

This experiment is designed much the same as the previous ones. The experimental design can be seen in Figure 3-11. 30 ml of innoculum, prepared as described in Section 3.3.1, was placed into six BOD bottles as described above. In the next step dilution water was placed into the BOD bottles. The dilution water was made up of either: 0 mg/L, 500 mg/L, or 1000 mg/L of each of the following nutrients: ammonium nitrate, potassium nitrate, and potassium dihydrogen phosphate in distilled water. The inorganic nutrients levels in the dilution are shown in Table 3-5. No attempt was made to alter the pH of the dilution water. The first three samples at each treatment level served as controls while the last three were dosed with either 4 ppm copper, 8 ppm cobalt, or 8 ppm zinc. The microcosms were then handled in the standard manner, Section 3.3.2.

	Cu (4 PPM)	Co (8 PPM)	Zn (8 PPM)
Low nutrient level			
Medium nutrient level			
High nutrient level			

Figure 3-11

Experimental Matrix Design for the Effect of Inorganic Nutrients on Metal Inhibition

Table 3-5
Nutrient Level for the Effects of Inorganic Nutrients on Metal Inhibition

	Low nutrient level (ppm)	medium nutrient level (ppm)	high nutrient level (ppm)
NH_4^+	0	244	488
NO_3^-	0	738	1476
$H_2PO_4^-$	0	468	936
K^+	0	214	428

3.5.7 The Effect of EDTA on the Metabolic Inhibition of Copper

This experiment consists of three sub experiments in which the effect of EDTA on copper inhibition is explored. In the first experiment EDTA was added in increasing amounts to samples dosed with a constant quantity of copper. The second experiment involved dosing a microcosm with copper, determining the rate of oxygen consumption, and then adding EDTA and seeing if there is any recovery as indicated by an increase in the rate of oxygen consumption. The third involved the addition of a premixed sample of EDTA and copper to determine the effect on the metabolism rate of a microcosm.

Scoping experiments were necessary to determine the potential toxicity of EDTA. Because it is a chelator it can possibly "steal" essential cations from the cell membranes of microorganisms destabilizing the membranes and causing toxicity. In the scoping experiments 30 ml aliquots of innoculum were placed in 300 ml BOD bottles as described in Section 3.3.1 and dilution water, prepared as described in Section 3.2.2, was added. Next varying amounts of EDTA were added. The microcosms were handled in the standardized manner as described in Section 3.3.2. The results of the metabolic inhibition of EDTA experiments are as shown in Table 3-6 .

Table 3-6

Metabolic Inhibition by EDTA on Microorganisms

	0	16	33	66	166	333	666	1000
EDTA Exp#1	1	1.16	1.21	1.17	1.19	1.19	-*	-*
EDTA Exp#2	1	.96	.95	.92	.84	.74	.507	.300
EDTA Exp#3	1	-*	-*	.982	.944	.964	-*	-*

-* No Data Collected

It was determined that EDTA up to 333 ppm could be used without significant inhibitory effects to the microorganisms.

3.5.7.1 Effect of increasing EDTA Concentration on Copper Inhibition

In the first experiment 6 samples were prepared with 30 ml of innoculum as described in Section 3.3.1, and dilution water prepared as described in Section 3.2.2 was added. Next varying amounts of EDTA, followed by a fixed amount of copper was added. Dilution water prepared as described in Section 3.2.2 was added and the microcosms were handled in the standard method as described in Section 3.3.2. In this experiment EDTA is added first followed by copper. They were added in the following proportions (Table 3-7):

Table 3-7

EDTA Effect on Copper Inhibition Experiment

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Cu (PPM)	0	0	33	33	33	33
EDTA (PPM)	0	333	0	66	166	333

3.5.7.2 Microorganism Recovery from Copper Inhibition by EDTA Addition

The second experiment involved the use of 150 ml of innoculum and 135 ml of dilution water prepared as described above. The two were combined in a 300 ml BOD bottle and toluene saturated water was added to bring the toluene concentration to 26 PPM. The BOD probe was placed in the bottle and the dissolved oxygen consumption was charted on a strip chart recorder set at 1 cm/min. recording speed and recording DO (mg/L) vs. time (min.). When a straight line slope was determined after approximately 5 minutes, 33 ppm Cu was added to the sample and the BOD probe replaced. Approximately five minutes later a straight line slope was determined. The BOD probe was removed and 10 ml of sample was removed and 10 ml of 10,000 ppm EDTA was added. The probe was replaced and a new slope determined.

After completion of the experiment the DO consumption of each treatment was determined by picking DO points off the graph and determining the elapsed time between them.

3.5.7.3 *Effect of Copper-EDTA Complex on Metabolic Inhibition*

The third experiment was set up similarly to the second except that after the initial DO consumption rate was determined a premixed solution of 10 ml of EDTA and 1 ml of 10,000 ppm Cu was placed into the BOD sample bottle after 11 ml of the innoculum/dilution water had been removed. The BOD probe was replaced and a post treatment slope traced. The slopes in this experiment were determined by the same method as described in the second EDTA experiment.

3.5.8 *Effect of Sodium Chloride on Metabolic Inhibition*

This experiment explores the changes in metabolic inhibition for copper, cobalt, and zinc for three treatments of sodium chloride. It is an attempt to determine if sodium chloride levels can effect the metabolic inhibition of a metal.

This experiment is designed much the same as the previous ones. The experimental design can be seen in Figure 3-12. 30 ml of innoculum, prepared as described in Section 3.3.1, was placed into six BOD bottles as described above. In the next step dilution water, prepared as described in Section 3.2.2, was placed into the BOD bottles. 0 mg/L, 166 mg/L, or 333 mg/L of sodium chloride was then added. The first three samples at each treatment level served as controls while the last three were dosed with 4 ppm of copper, cobalt, or zinc. The microcosms were then handled in the standard manner, Section 3.3.2. Two replicates of the experiment were completed with new batches of innoculum.

	Cu	Co	Zn
0 ppm Cl^-			
166 ppm Cl^-			
333 ppm Cl^-			

Figure 3-12

Experimental Matrix Design for the Effect of Sodium Chloride on Metal Inhibition

3.6 Adsorption Experiments Using X-ray Flourescence Analysis

In this part of the thesis x-ray analysis will be used to determine the relative quantity of metal adsorbed on microorganisms. The equipment used is the Baird 6500 Energy Dispersive XRF spectrometer. These experiments are an attempt to correlate the amount of metal adsorbed on a microorganism to the toxicity exhibited by the microorganisms.

3.6.1 X-ray Theory (Skoog and Leary, 1992:357-381)

X-ray analysis uses x-rays of energies between 124 keV and 50 keV depending on the source and what is to be excited. For x-ray generation there exists 2 types of x-ray spectra, continuous or Brensstrahlung (Figure 3-13)and line spectra (Figure 3-14). Continuous x-rays consist of a broad band of energies emitted from a source while line sources contain specific energy quanta.

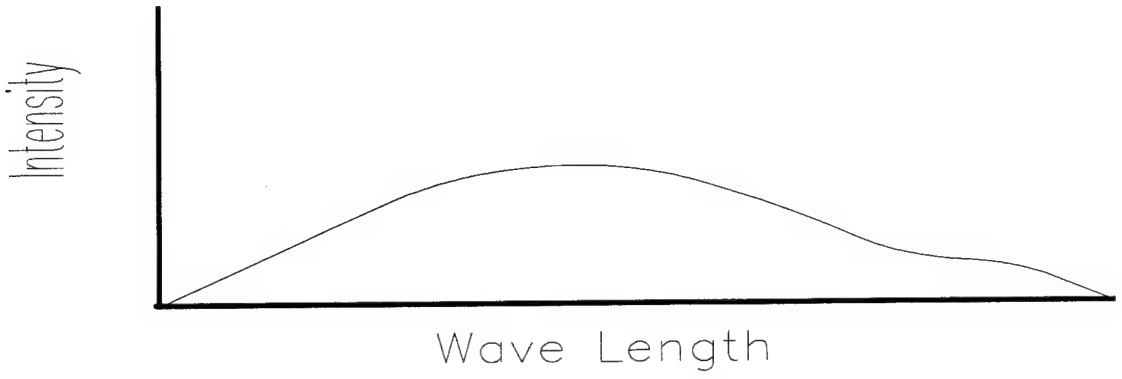


Figure 3-13
Continuous X-ray Spectra

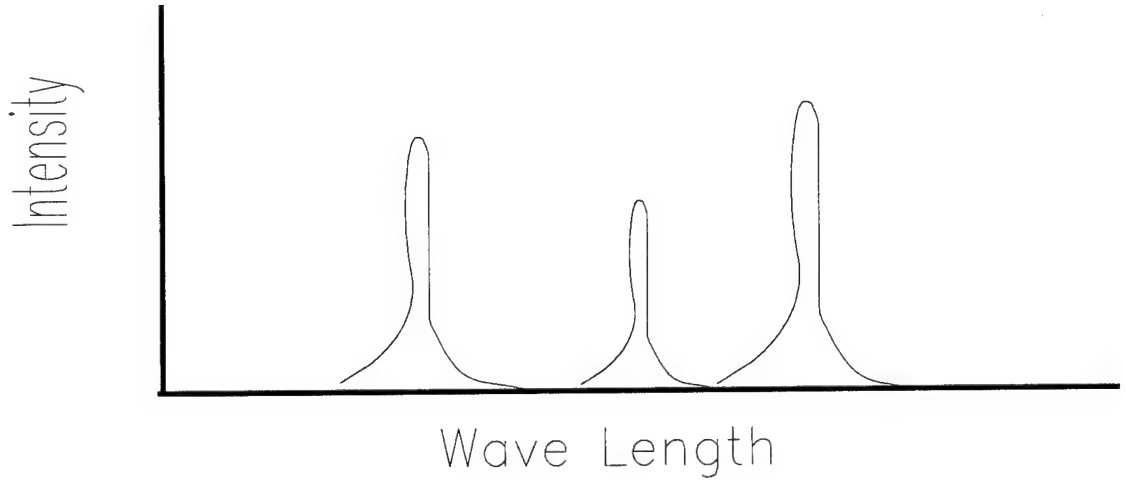


Figure 3-14
Line X-ray Spectra

The continuous spectrum has a short wavelength limit λ_0 that is a function of the electron accelerating voltage in the x-ray source and independent of the target material. The λ_0 is the highest energy of photon that the x-ray source can produce. It is the result of a perfectly inelastic collision between the electron and a target atom. For other collisions the electron loses part of its energy with each collision. The energy that is lost

excites target electrons to higher energy states and as these electrons relax a photon may be emitted. The photon has the energy equal to the difference in electron energy before and after the collision.

$$h \cdot \upsilon = E_1 - E_1' \tag{3.4}$$

The x-rays are emitted through an x-ray tube (Figure 3-15). In an x-ray tube a tungsten filament is heated to an extremely high temperature causing it to give off electrons. These electrons are accelerated across a potential and strike a target compound (in this experiment it is a rhodium target) which ejects an inner shell electron (Figure 3-16). This hole will be filled by electrons falling from upper shells. As the electrons fall into the inner shells x-rays are emitted. Because of the large energy difference between inner shells, the energy given off by the transition of electrons to the lower energy levels is large.

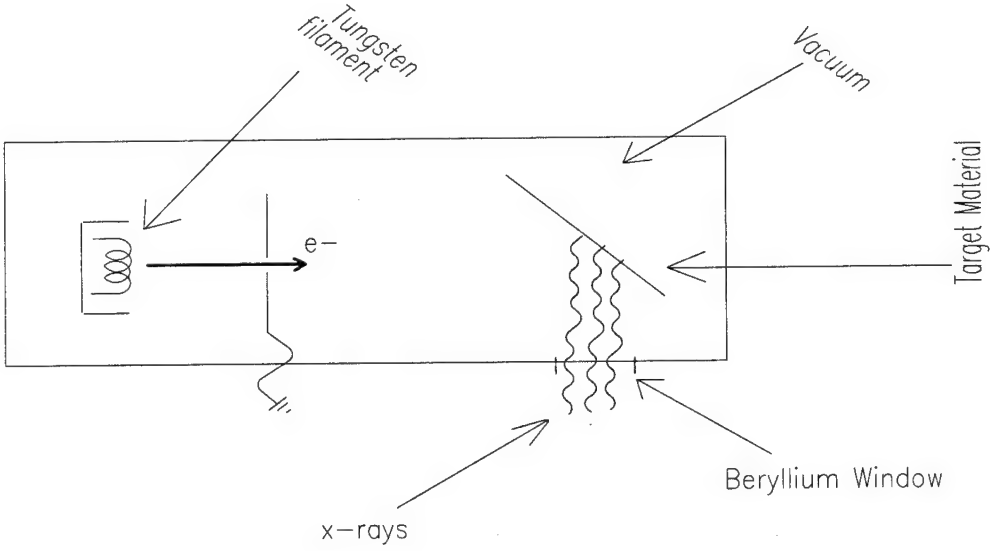


Figure 3-15
Schematic of an X-ray Tube

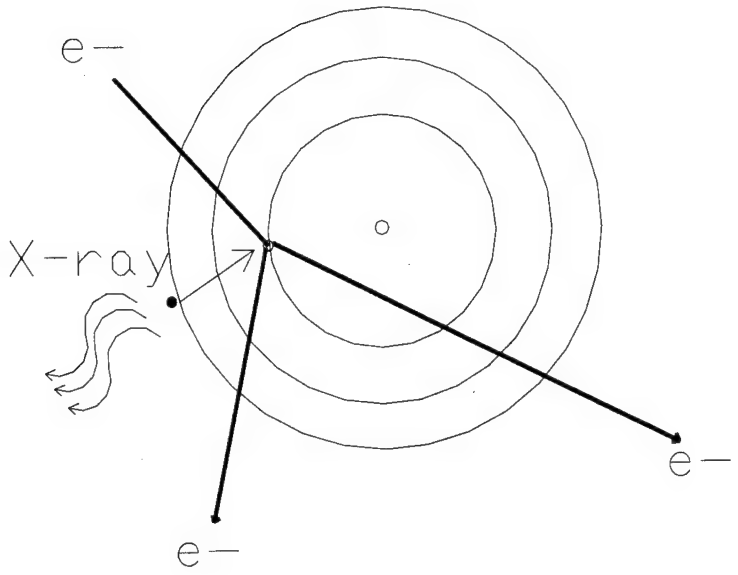


Figure 3-16

Representation of an Electron Being Kicked Out of Orbit

The x-rays emitted from the x-ray tube are directed at the sample. The high energy x-rays are able to remove electrons from the sample analytes and the electrons of the analyte emit x-rays of their own as the electrons cascade to lower energy levels (figure 3-17).

X-ray emission lines are remarkably simple when compared to UV or IR spectra and consist of only a few series of lines. The shorter wavelength groups called K series and longer ones called the L series. Elements with an atomic number less than 23 produce only the K series. These wavelengths (energies) produced are unique to the element and allow for identification and quantification.

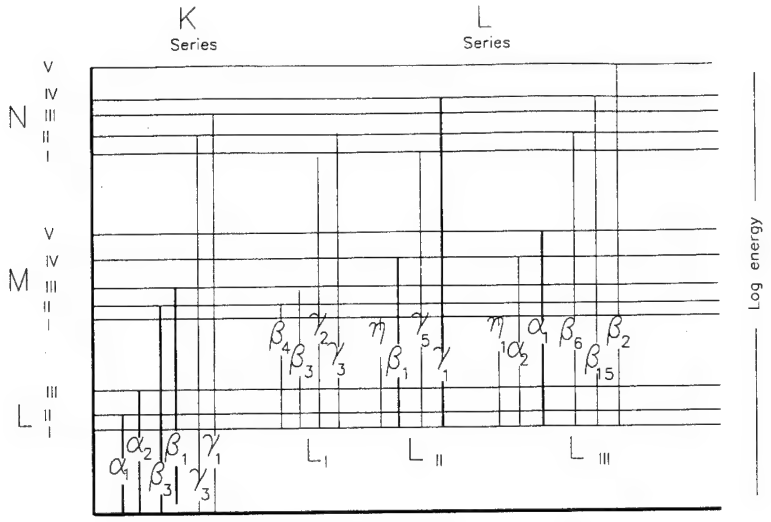


Figure 3-17
Electron Energy Level Diagram

The x-rays from the tube are directed through the beryllium window and onto the sample. These x-rays strike the sample and in turn may eject an electron from the sample material; as electrons cascade into lower energy levels more x-rays may be created by the sample. These x-rays are representative of the elements in the sample and can be used to identify elements in the sample. The process of using the x-ray spectra from the sample to identify and quantify elements in a sample is called x-ray spectroscopy.

In order to identify and quantify the elements present in a sample, the energy of the x-rays produced must be measured and the number of photons emitted by the sample counted. There exists two types of detectors, wavelength dispersive and energy dispersive. Wavelength dispersive instruments use a crystal to separate the x-rays into mono-chromatic wavelengths. The monochrometer sweeps through a range of wavelengths while the detector counts the number of photons at each wavelength. This type of instrument was not used in this experiment and will not be discussed further.

The second type of detector is an energy dispersive detector. In this case no monochrometer is used, instead a lithium drifted silicon detector is used to absorb all the x-rays that strike the detector. Initial absorption of a photon results in the formation of a highly energetic photoelectron which then loses its kinetic energy by elevating thousands of electrons in the silicon conductor band, a marked increase in conductivity results. When a potential is placed across the detector a current pulse results. The size of the pulse is directly proportional to the energy of the absorbed photon. The signal is output to two amplifiers, a fast and slow amplifier. The fast amplifier tells whether or not two photoelectrons are striking the detector at the same time. If this is occurring the signal is locked out. The period of time that the system is locked out is called dead time. This usually should be kept below 40%. If only one photon is hitting the detector the slow amplifier amplifies it and determines its intensity. It is then registered as a count at the electron energy that created the pulse. The counts are normalized to 100 or 1000 and a histogram presented over the energy levels. This histogram represents the spectrum.

Calibration curves can be generated from standards to determine the quantity of a substance in a sample; however matrix effects become very important and a standard must match the sample matrix as closely as possible. A second method exists that gives a semi-quantitative analysis without the use of standards. It is called standardless fundamental parameters. It works by knowing the relative x-ray fluorescence yield intensities for each element and all the interactions between x-rays and matrix atoms and uses the acquired spectra to solve a multi-variable system to arrive at a best fit of the

quantities of a substance to the spectrum. Matrix corrections for very thin samples compared to the x-ray range are small.

3.6.2 Standardizing the Quantity of Microorganisms and Metals in Samples

Unfortunately there were no standards available for microorganisms and metal content on filter paper, this precluded the use of standards to determine the quantity of metals on the microorganisms. The quantity of elements that are not detectable by the XRF machine was unknown so the standardless fundamental parameters could not properly be used to determine the quantity of microorganisms and metal on the microorganisms. This made absolute quantification very difficult to obtain. It was therefore determined that differences in metal adsorption would be examined. First an inorganic element that was representative of living microorganisms was necessary. Normalizing to the quantity of this element would account for uneven distribution of microorganisms on the filter paper surface, as well as, differences in the quantity of microorganisms in the samples. Phosphorous was chosen as the element most closely related to population size; scoping studies varying the quantity of microorganisms produced a linear increase in the number of phosphorous counts when examined on the x-ray spectrometer. The results of this experiment can be seen in Figures 3-18.

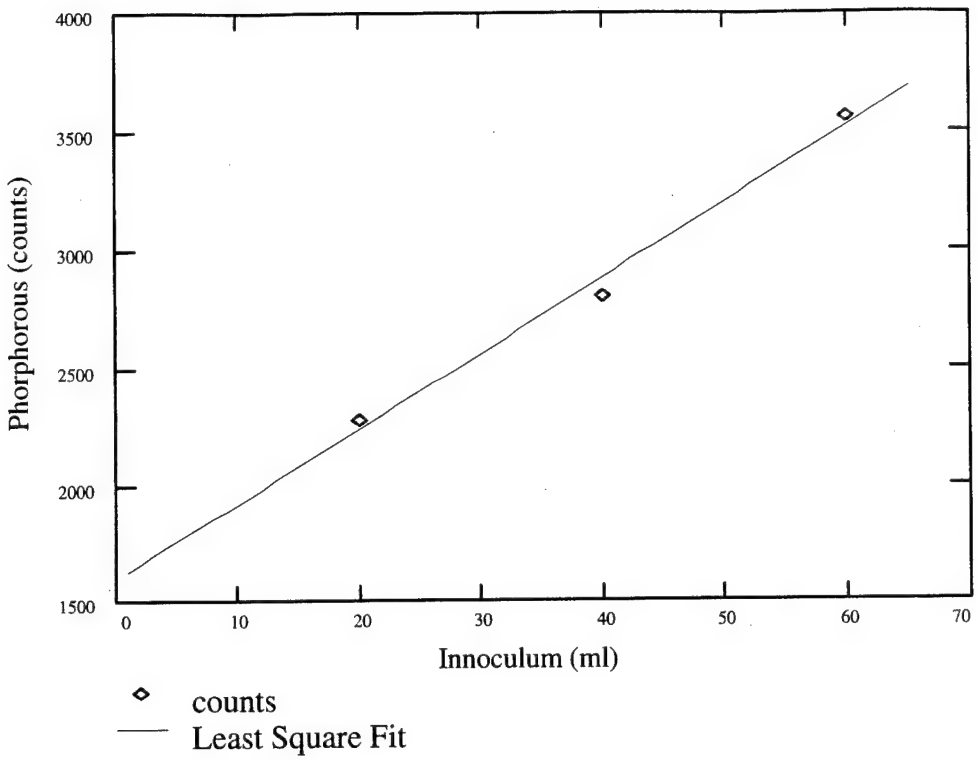


Figure 3-18

Correlation of Phosphorous Counts and Microbial Population

showing that the peak area of phosphorous is proportional to the quantity of microorganisms

If phosphorous is indeed representative of the microbial population and the metals adsorb homogeneously to the microorganisms surface, then testing completed on different quantities of metal-dosed microorganisms should show an increase in metal counts that is proportional to the increase in phosphorous counts. It was shown in further scoping experiments that phosphorous and copper counts increased proportionally as the quantity of microorganisms examined by x-ray fluorescence was increased. See Figure 3-19 for results.

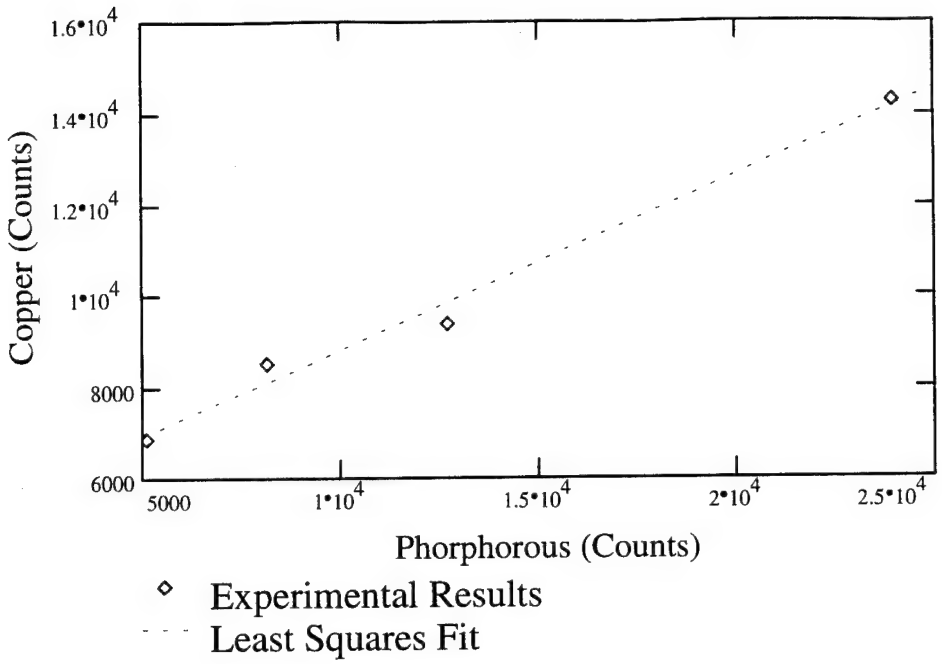


Figure 3-19

Correlation of Copper with Phosphorous Counts as the Quantity of Microorganisms was Increased

Showing that for different quantities of metal dosed microorganisms the peak area of metal is linearly related to the peak area of phosphorous

Confident that the ratio of metal counts to phosphorous counts was a valid indicator of the quantity of metal on a microorganism, certain experiments were repeated and the microorganisms from these experiments examined using x-ray fluorescence to determine if a significantly different metal to phosphorous count ratio occurred. This would indicate that different quantities of metal were adsorbed to the microorganisms; differences in adsorption would then be correlated to differences in toxicity.

3.6.3 Standard X-ray Fluorescence Analysis

Just as metabolic inhibition was measured in a standardized manner, adsorption was also measured in a standardized manner. Experiments mimicking the inhibition experiments were prepared; the experiments will be described in further detail in later sections. 100 ml aliquots of the treated microcosms were filtered through 1.2 micrometer Millipore® filters that had been previously tarred. The filters where allowed to dry at room temperature for two days before being weighed again. The difference in the two weights represents the weight of both the microorganisms and metals.

The samples were then placed in x-ray fluorescence sample holders and covered with .6 mm mylar film. The samples are placed in the Baird 6500; each sample is examined under a vacuum at an x-ray tube voltage of 20 kv and a tube current of 250 milliamps for a live count time of 600 seconds. The analysis is completed with the 4 mm collimator, the x-ray energy levels are monitored at the detector over the 0 to 10 kv range and no filters or secondary targets are used.

The analysis produces a histogram of the number of counts at each energy; the computer software is used to automatically identify the elements and the number of counts in a predetermined peak area for each element. The peak areas for the metal of interest and phosphorous are then used to calculate the ratio of metal to phosphorous. If these ratios are significantly different between differing treatments, then different quantities of metal were adsorbed by the microorganisms. The treatment with the higher ratio will have more of the metal adsorbed.

$$Ratio = \frac{peak \cdot area(metal)}{peak \cdot area(phosphorous)} \tag{3.5}$$

3.6.4 Effect of pH on Metal Adsorption by Microbes

In this experiment the effects of pH on metals adsorption by microorganisms was investigated. Ten samples were prepared as described in Section 3.5.2 for each of three metals, copper, cobalt, and zinc. One sample served as a control at pH 7 and received no metal, while three were filled with dilution water at pH 5, three with dilution water at pH 7, and three with dilution water at pH 9. Each was then dosed with 10 ppm of the metal of interest.

Starting with a pH 5 microcosm, then pH 7 and finally pH 9, the microcosms were handled and analyzed as described in Section 3.6.4. After all pH treatment levels were tested, the process was repeated two more times for the replicates. The peak areas were determined and the ratio of metal to phosphorous counts calculated. If no peak for the metal was discernible by the computer software, then the metal was assumed not to be present in the sample. The result were next tabulated, graphed, and analyzed to determine if a difference in means had occurred.

3.6.5 The Effect of Mg on the Adsorption of Metals by Microorganisms

In this experiment the effects of increasing levels of magnesium on metals adsorption by microorganisms was investigated. Nine samples were prepared as described in Section 3.5.3 for each of three metals, copper, cobalt, and zinc. Three received no additional magnesium, three received 166 ppm additional calcium and three

received 333 ppm additional magnesium. Each was then dosed with 10 ppm of the metal of interest.

Samples from the microcosms were filtered through millipore filters by the same procedure described in section 3.6.4. The treatment levels were analyzed in the following order: 0 ppm, 166 ppm, and 333 ppm additional magnesium respectively. After all magnesium treatment levels were tested, the process was repeated two more times for the replicates. The peak areas were determined and the ratio of metal to phosphorous counts determined. If no peak for the metal was discernible by the computer software, then the metal was assumed not to be present in the sample. The result were next tabulated and graphed to determine if a difference in means had occurred.

3.7 Statistics

This section will describe the statistics that are used to assess the significance of the results which have previously been obtained. This section will describe the mean, standard deviation, normality of data, analysis of variance, and linear regression. The first step in analyzing the data was to determine the mean and standard deviation. These values were tabulated and presented in the results. Next an Anova was conducted to determine the statistical significance of the results.

3.7.1 Mean

The mean of a set of numbers is simply the arithmetic average of that set. It is also referred to as the sample mean ($\overline{X}$) and is determined by the formula (Devore, 1991:15).

$$\overline{X} = \frac{X_1 + X_2 + ... + X_n}{n} \tag{3.6}$$

$\overline{X}$ represents an unbiased estimator of the population mean μ, the expected value of the population. Because the sample space of results is infinite, μ can never truly be known so it must be estimated with $\overline{X}$ our best estimate.

3.7.2 Standard deviation

The sample standard deviation (s) is used as an indicator of the variability in a sample from a population. It is determined by the equation:

$$s^2 = \frac{\sum_{i=1}^{n} (X_i - \overline{X})^2}{n-1} \tag{3.7}$$

This variable includes the natural population variance as well as instrument variability and other measurement uncertainties. A small value of standard deviation means that most values are tightly bound around the mean indicating low variability while a larger s indicates greater variability.

3.7.3 Normality

The Anova analysis performed later in this section requires that the values from the population to be normally distributed. Normality is indicated by a probability distribution "which is symmetric about μ and bell shaped so the center of the bell is both the mean of the distribution and the median. The value of σ is the distance from μ to the inflection points on the curve (the points at which the curve changes shape from turning

downward to turning upward)". (Devore, 1991:144) Normality will be tested with the Wilk-Shapiro statistic and the Rankit plot. A Wilk-Shapiro statistic over .8 will be considered normally distributed (Devore, 1991).

3.7.4 Analysis of Variance (Netter and others, 1991)

Analysis of variance is a set of statistical procedures to analyze the relation between a dependent variable and one or more independent variables. In this thesis the dependent variable is the normalized metabolic activity rate or the ratio of metal counts to phosphorous counts. An independent variable, that is altered during an experiment is called a factor. Each factor under investigation was tested at three levels (low, medium and high or pH 5, pH 7 and pH 9). Two factors can be investigated at one time. Any combination of two distinct factor levels is called a treatment or cell. It is into each cell that the toxicity data is placed.

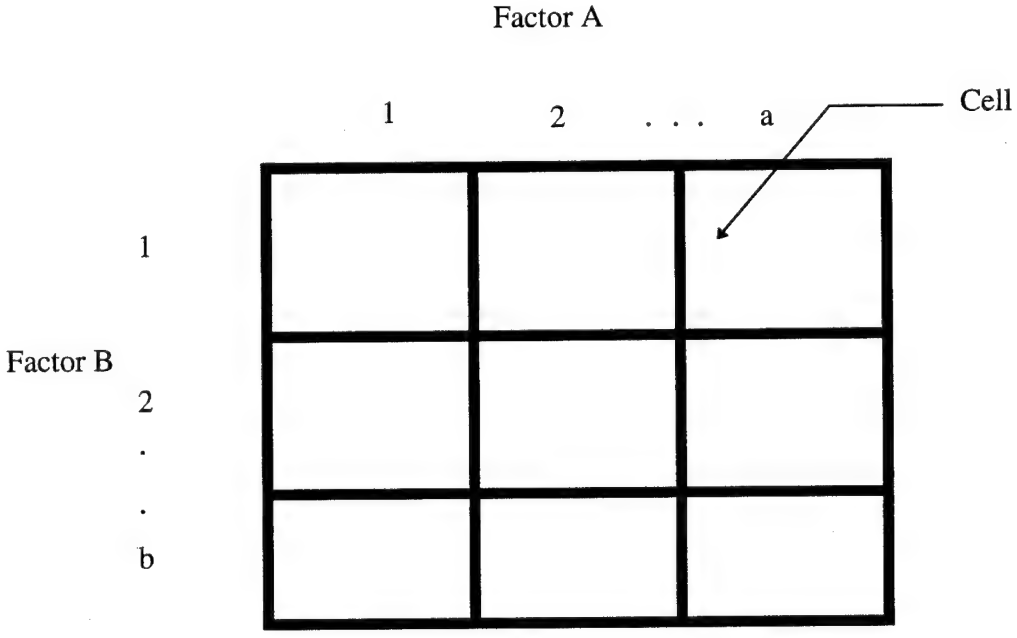


Figure 3-20
Sample Two Factor Experiment Design

Such a multi-factor study offers advantages over several single factor studies; it is more efficient because it allows for the study of many factors at once. In addition it strengthens the validity of the findings by investigating factors of possible secondary importance to permit inferences about the primary factors with a greater range of validity. A flow chart of the Anova process as it is used in this thesis is provided in figure 3-21.

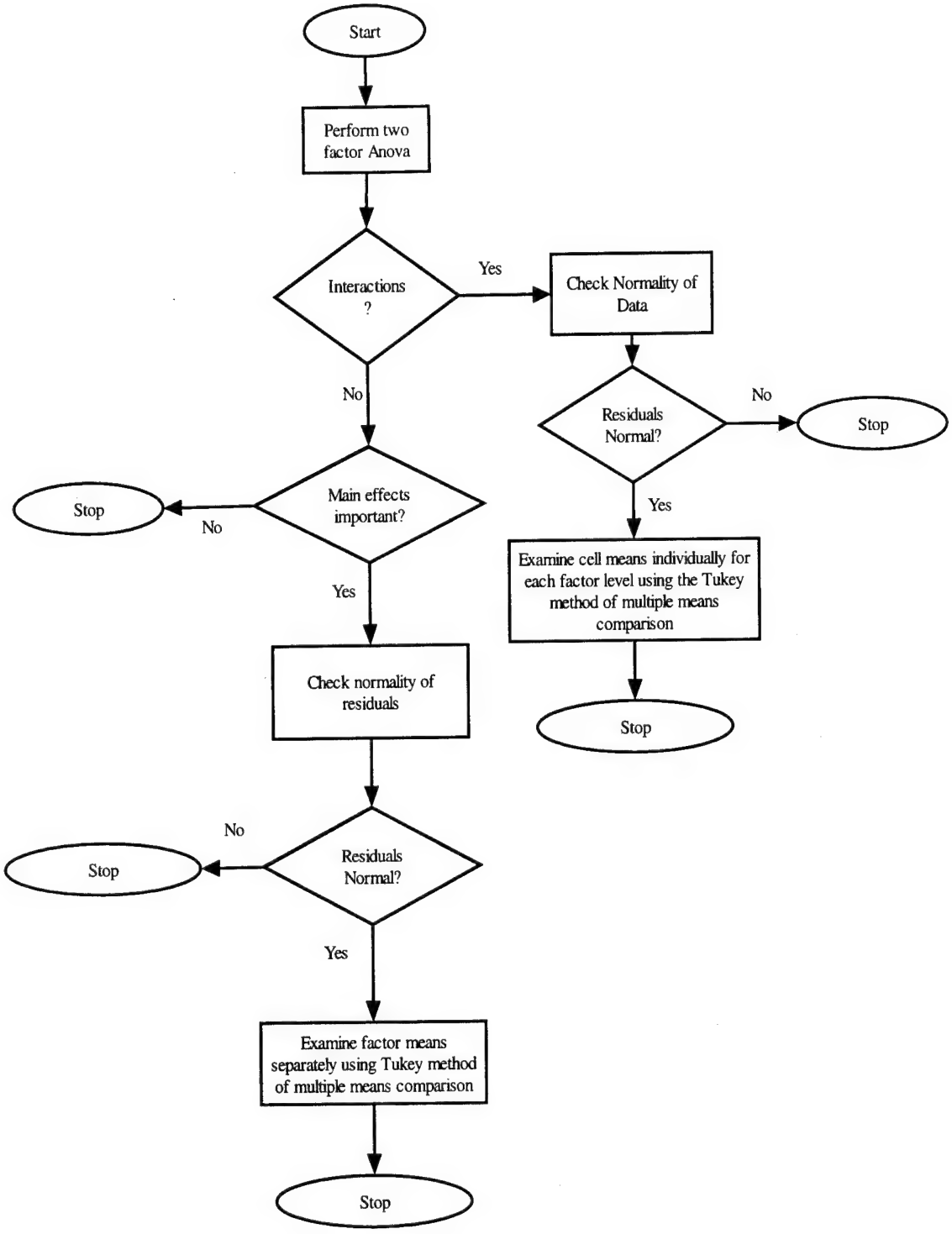


Figure 3-21

Flow Chart for the Analysis of Variance

Describes the steps in the statistical analysis of experimental results

3.7.4.1 Treatment Means

The mean toxicity response for a treatment in a two factor study is given by μ_{ij} where i is the factor level of factor A and j the factor level of factor B. The factor level mean is quite simply the average of the row and column treatment means, the column factor level means are denoted by $\mu_{\bullet j} = \frac{\sum_{i=1}^{a} \mu_{ij}}{a}$ and the row factor means are denoted by $\mu_{i\bullet} = \frac{\sum_{j=1}^{b} \mu_{ij}}{b}$ the overall mean is denoted by $\mu_{\bullet\bullet} = \frac{\sum_{j=1}^{b}\sum_{i=1}^{a} \mu_{ij}}{a \cdot b}$. $\mu_{\bullet j}$ denotes the mean of treatment means of the j^{th} factor level of factor B, while $\mu_{i\bullet}$ is used to indicate the mean treatment level mean of the i^{th} factor level of factor A. $\mu_{\bullet\bullet}$ is the overall mean.

3.7.4.2 Main Effects

The main effect of a treatment is the difference between the factor level mean and the overall mean for the columns (Factor A) $\alpha_i = \mu_{i\bullet} - \mu_{\bullet\bullet}$ and rows (Factor B) $\beta_j = \mu_{\bullet j} - \mu_{\bullet\bullet}$.

3.7.4.3 Additive Factor Effect

The factors may be checked for interactions through the use of additive factor effects. If for every μ_{ij}; $\mu_{ij} = \mu_{\bullet\bullet} + \alpha_i + \beta_j$ then we can claim that the factors do not interact or that no factor interaction has occurred. The significance of this is that the

factors may now be described separately by analyzing the factor level means or the factor main effects.

Whether or not factors have any interaction can also be determined if the difference between the mean response for any two levels of factor B is the same for every A factor level and vise versa for factor A at every level of factor B. If the curves of the mean responses for factor levels are parallel no factor interactions are present as exemplified in Figure 3-22.

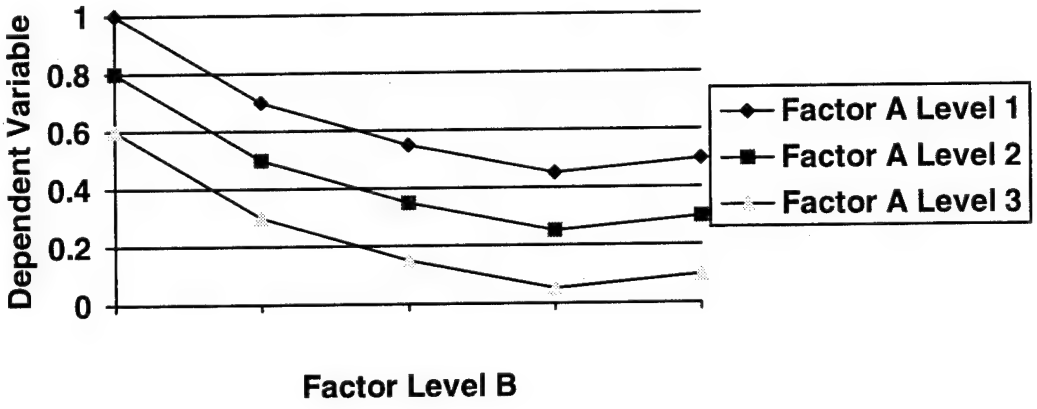


Figure 3-22
Graphical Test for Interaction in a Two Factor Anova
Parallel lines indicate that there is no interaction between factor levels

3.7.4.4 Fixed Factor Level Study

This is a fixed factor level study because the factor levels are chosen because of intrinsic interest in them and because they are not considered as a sample from a larger population. In this model $Y_{i,j,k} = \mu_{i,j} + \varepsilon_{i,j,k}$ where μ_{ij} is the mean of the i,j th treatment population and ε_{ijk} is the error associated with the Y_{ijk} th observation due to

population variance and measurement errors. If several observations are made of the same treatment an average may be obtained that is the unbiased estimator of the population mean. If observations are made for every factor in the study them all the treatment means, factor level means, and the overall mean may be computed.

It is possible to break up the results of a factor mean into its constituent parts; thus it is possible to estimate the effects of factor A and factor B on a treatment as well as the effect of the interactions of the two factors. The purpose of a two factor Anova is to determine to a level of statistical significance if factor A, factor B or an interaction of factor A and B have an effect. This is accomplished by a mathematical process described in the next section.

$$\overline{Y}_{i,j,\bullet} - Y_{\bullet\bullet\bullet} = (\overline{Y}_{i\bullet\bullet} - \overline{Y}_{\bullet\bullet\bullet}) + (\overline{Y}_{\bullet j\bullet} - \overline{Y}_{\bullet\bullet\bullet}) + (\overline{Y}_{i,j,\bullet} - \overline{Y}_{i\bullet\bullet} - \overline{Y}_{\bullet j\bullet} + Y_{\bullet\bullet\bullet})$$

Main Effect A | Main Effect B | A*B Interaction

3.7.4.5 *Performing an Anova by Hand*

The following is a step by step description of how to complete an Anova. In this thesis effort the Anova analysis was done using *Statistix 4.1*. In the first step treatment means, factor means, and overall means are computed.

$$\overline{Y_{i,j}} = \frac{\sum_{k=1}^{N} Y_{i,j,k}}{N}$$

$$\overline{Y_{i\bullet\bullet}} = \frac{\sum_{j=1}^{b}\sum_{k=1}^{N} Y_{i,j,k}}{b \cdot N}$$

$$\overline{Y_{\bullet j\bullet}} = \frac{\sum_{i=1}^{a}\sum_{k=1}^{N} Y_{i,j,k}}{a \cdot N}$$

$$\overline{Y_{\bullet\bullet\bullet}} = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{N} Y_{i,j,k}}{a \cdot b \cdot N}$$

Where:

$Y_{i,j,k}$ is the k^{th} observation of treatment i,j

i treatment factor A

j treatment factor B

a,b,N is the number of A factor levels, B factor levels and replications of treatments respectively

Next the total sums of squares (SSTO) is computed. This may be broken up into sums of squares between treatments and sums of squares within treatments; these are called SSTR and SSE. The treatment sum of squares may be further broken down into sums of squares arising due to factor A effects, factor B effects and A*B interactions. These sums of squares are called factor A sum of squares (SSA), factor B sum of squares (SSB), and AB interactions sum of squares (SSAB). The equations to compute the sums of squares used in this analysis are given below:

$$SSTO = \sum_i \sum_j \sum_k (Y_{i,j,k} - Y_{...})^2$$

$$SSTR = N \cdot (\sum_i \sum_j (Y_{i,j,\bullet} - Y_{...})^2)$$

$$SSE = \sum_i \sum_j \sum_k (Y_{i,j,k} - Y_{i,j,\bullet})$$

$$SSTR = SSA + SSB + SSAB$$

Where:

$$\text{SSA} = N \cdot b \sum_i (\overline{Y}_{i\bullet\bullet} - \overline{Y}_{...})^2$$

$$\text{SSB} = N \cdot a \sum_j (\overline{Y}_{\bullet j\bullet} - \overline{Y}_{...})^2$$

$$\text{SSAB} = N \cdot \sum_i \sum_j (\overline{Y}_{ij\bullet} - \overline{Y}_{i\bullet\bullet} - \overline{Y}_{\bullet j\bullet} + \overline{Y}_{...})^2$$

The sum of squares errors are then divided by their appropriate degrees of freedom to obtain the associated mean square errors. It is these values that are used to compute the F statistic that checks for significant significance.

$$MSA = \frac{SSA}{a-1}$$

$$MSB = \frac{SSB}{b-1}$$

$$MSE = \frac{SSE}{a \cdot b \cdot (n-1)}$$

$$MSAB = \frac{SSAB}{a \cdot b \cdot (N-1)}$$

In order to complete an Anova one must have a null and alternative hypothesis. The null hypothesis is rejected if one or more of the treatment means under analysis are different.

The first test in a two way Anova is to look for interactions. For this test the following hypothesis is put forward:

$H_o: \mu_{ij} - \mu_{i\bullet} - \mu_{\bullet j} + \mu_{\bullet\bullet} = 0$

$H_a: \mu_{ij} - \mu_{i\bullet} - \mu_{\bullet j} + \mu_{\bullet\bullet} \neq 0$

The null hypothesis claims that there are no interactions between factors while the alternate claims that there is significant interaction between factors. If the null is rejected in favor of the alternate then significant interactions exist between factors A and B and there is no need to check for A or B main effects. If significant interactions are found between factors then Tukey means comparisons are performed on all pairwise differences of the treatment means $\mu_{i,j}$.

The appropriate test statistic for interaction is $F^* = \frac{MSAB}{MSE}$. The test rule for this test is:

If $F^* \leq F[1-\alpha;(a-1)\cdot(b-1),(n-1)\cdot a\cdot b]$ accept H_o

If $F^* > F[1-\alpha;(a-1)\cdot(b-1),(n-1)\cdot a\cdot b]$ reject H_o

If there is no significant interaction the next test should examine whether or not a statistically significant difference exists between one or more treatment level means of each factor A and B. If significant difference exist among factor A or B level means then a Tukey means comparison can be employed to determine which factor level means are statistically significantly different.

To test factor A or factor B level means the following hypothesis is put forward:

H_o:$\mu_{1\bullet} = \mu_{2\bullet} = \mu_{a\bullet}$ Or H_o:$\mu_{\bullet 1} = \mu_{\bullet 2} = \mu_{\bullet b}$

H_a:$\mu_{1\bullet} \neq \mu_{2\bullet} \neq \mu_{a\bullet}$ H_a:$\mu_{\bullet 1} \neq \mu_{\bullet 2} \neq \mu_{\bullet b}$

The null hypothesis states that there are no main factor A or B effects while the alternate states that main factor effects occur. The appropriate test statistic is:

$F^* = \frac{MSA}{MSE}$ or $F^* = \frac{MSB}{MSE}$. The decision rule for either of these tests is:

If $F^* \leq F[1-\alpha;(a-1)\cdot(b-1),(n-1)\cdot a\cdot b]$ accept H_o

If $F^* > F[1-\alpha;(a-1)\cdot(b-1),(n-1)\cdot a\cdot b]$ reject H_o

3.7.5 Tukey Method of Multiple Means Comparison

In this paper we will be using the Tukey method with equal sample size to study the statistical significance of differing levels of treatment means. From this information conclusions may be drawn as to whether the results between factor levels come from different populations.

The Tukey procedure uses the Studentized range distribution to compute a "collection of simultaneous confidence statements about the true values of all differences (μ_1-μ_2) between true statements means" (Devore, 1991:381) .

The confidence interval is given by the following equation:

$$\overline{X_1} - \overline{X_2} - Q_{\partial,k,k(k-1)} \cdot \sqrt{\frac{MSE}{2}\cdot(\frac{1}{k}+\frac{1}{k})} \leq \mu_i - \mu_j \leq \overline{X_i} - \overline{X_j} + Q_{\partial,k,k(k-1)} \cdot \sqrt{\frac{MSE}{2}\cdot(\frac{1}{k}+\frac{1}{k})}$$

Where:

$\overline{X}_1$, $\overline{X}_2$ = the means of each statement

α = experimental wise error rate

K = number of samples from each treatment

MSE = mean square error (for this experiment we will use the family mean square error computed in the two way Anova.

Q = Studentized range statistic

In practice the Tukey method is performed by computing a half width confidence interval and comparing it's value to all pairwise differences of sample means. Any difference of means that is greater than the half width interval is declared statistically significantly different. A simple algorithm to implement this process is as follows: 1. The half width interval is given by Eq 3-8.

$$Q_{\alpha,I,I,I\cdot(J-1)} \cdot \sqrt{\frac{MSE}{2} \cdot (\frac{1}{k} + \frac{1}{k})} \tag{3.8}$$

Next the computed sample means of treatments in question are listed in ascending order. All the means are compared to each other; and if

$\left|\overline{X}_1 - \overline{X}_2\right| < Q_{\alpha,I,I,I\cdot(J-1)} \cdot \sqrt{\frac{MSE}{2} \cdot (\frac{1}{k} + \frac{1}{k})}$ then the two means are not considered statistically significantly different and a line is drawn under them.

3.7.6 Least Squares Fit (Normal Error Regression Model)

In least square fit, an attempt to fit the best straight line through a set of data is made. It provides an unbiased estimator of β_0 and β_1 and produces the smallest sum of squares. The normal error regression model is given by

$$Y_i = \beta_0 + \beta_1 \cdot X_i + \varepsilon_i$$

Where:

Y_i is the observed response on the i^{th} trial

X_i is the level of the independent variable of the i^{th} trial.

β_0, β_1 are parameters of the model

ε_i is the error term and is independent $N(0,\sigma)$

Since the $E(\varepsilon)=0$ the expected value of Y_i at X_i is equal to

$E(Y_i / X_i) = \beta_0 + \beta_1 \cdot X_i$ and for any given independent variable

$\hat{Y} = \widehat{E(Y_i / X_i)} = \beta_0 + \beta_1 \cdot X$. The parameters β_0 and β_1 must be determined. This is done by selecting values of β_0 and β_1 that minimize the square of the residual. The residual is defined as the difference between observed Y_i and its estimate Y_i. Residuals are shown graphically in figure 3-23.

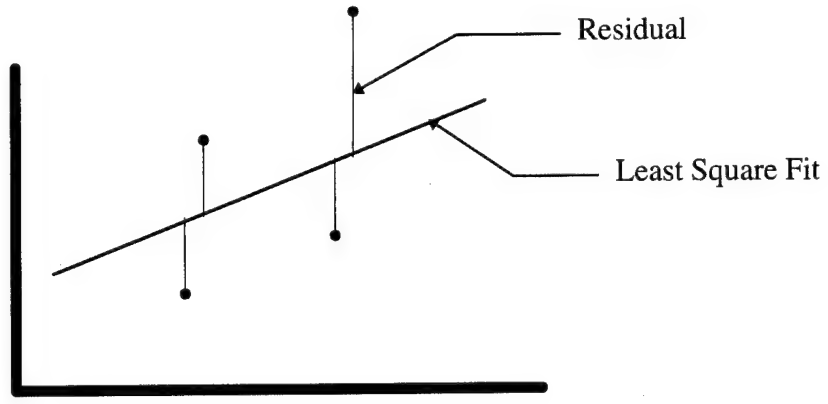


Figure 3-23
Pictorial Representation of Residuals and Least Squares Fit

In order to determine the parameters of the model three quantities S_{xx}, S_{yy}, and S_{xy} must be calculated. There equations are given below:

$$S_{xx} = \sum (X_i - \overline{X})^2$$
$$S_{yy} = \sum (Y_i - \overline{Y})^2$$
$$S_{xy} = \sum (X_i - \overline{X}) \cdot (Y_i - \overline{Y})$$

The slope (β_1) and intercept (β_0) of the best fit line are given by the equations:

$$\beta_1 = \frac{S_{xy}}{S_{xx}}$$

$$\beta_0 = \overline{Y} - m \cdot x$$

For this thesis effort, once the parameters for the least square fit are determined the estimator Y- will be plotted with observed values Y_i and a judgment call will be made as to whether or not the observed values are linear.

3.7.6 Example of Statistical Analysis

This section will present an example of the statistical analysis conducted in this thesis effort using use the results of the pH experiment. First an Anova will be conducted, the normality of the residual was checked and a Tukey comparison of means was conducted.

Results of the pH experiment:

	pH5 mean	pH5 Std	pH7 mean	pH7 Std	pH9 mean	pH9 Std
Cu	.486	.215	.201	.039	.3680	.195
Co	.839	.150	.7317	.071	.715	.154
Fe	.528	.0927	.932	.113	1.019	.011
Mn	1.043	.0212	.9707	.098	.9707	.104
Zn	.8253	.179	.5167	.051	.8477	.172

Plot of Results to visually check for interactions Figure 3-24 . The lines are not parallel; this is indicative of interaction between treatment factors. Next a two way Anova was accomplished with Statistix 4.1. The results are presented in Figure 3-25 .

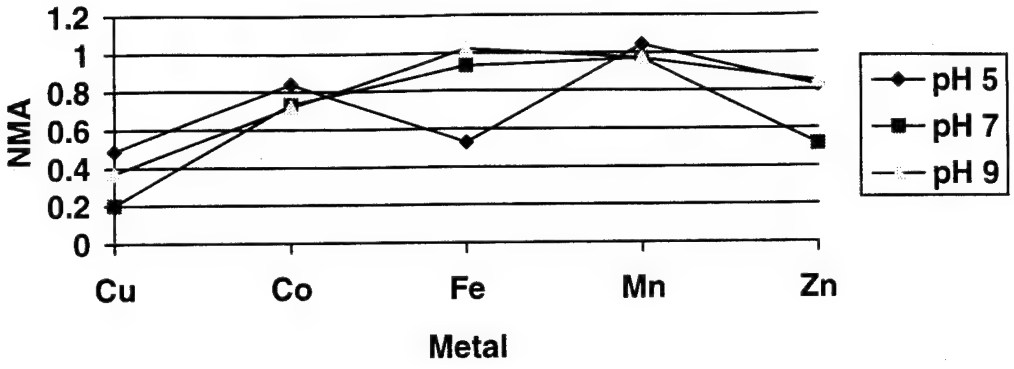


Figure 3-24

Plot of pH Results

Parallel lines would indicate no interaction. The lines are not parallel indicating that there is interaction.

STATISTIX 4.1 PHTOX1, 11/03/95, 10:37

ANALYSIS OF VARIANCE TABLE FOR TOXICITY

SOURCE	DF	SS	MS	F	P
METAL (A)	4	2.00602	0.50150	59.83	0.0000
PH (B)	2	0.09927	0.04964	5.92	0.0068
A*B	8	0.67638	0.08455	10.09	0.0000
RESIDUAL	30	0.25145	0.00838		
TOTAL	44	3.03312			

Figure 3-25
Results of Statistix Two Way Anova

First the normality of the residuals must be determined. The residuals were calculated and saved in statistix and plotted on a Rankit plot Figure 3-26. The Wilk Shapiro was then calculated to be .9867 indicating that the residuals are normally distributed. The small p value associated with the A*B interaction term indicates that

there is interaction between factors. This lead to an analysis of the treatment means μ_{ij} using the Tukey multiple comparison method.

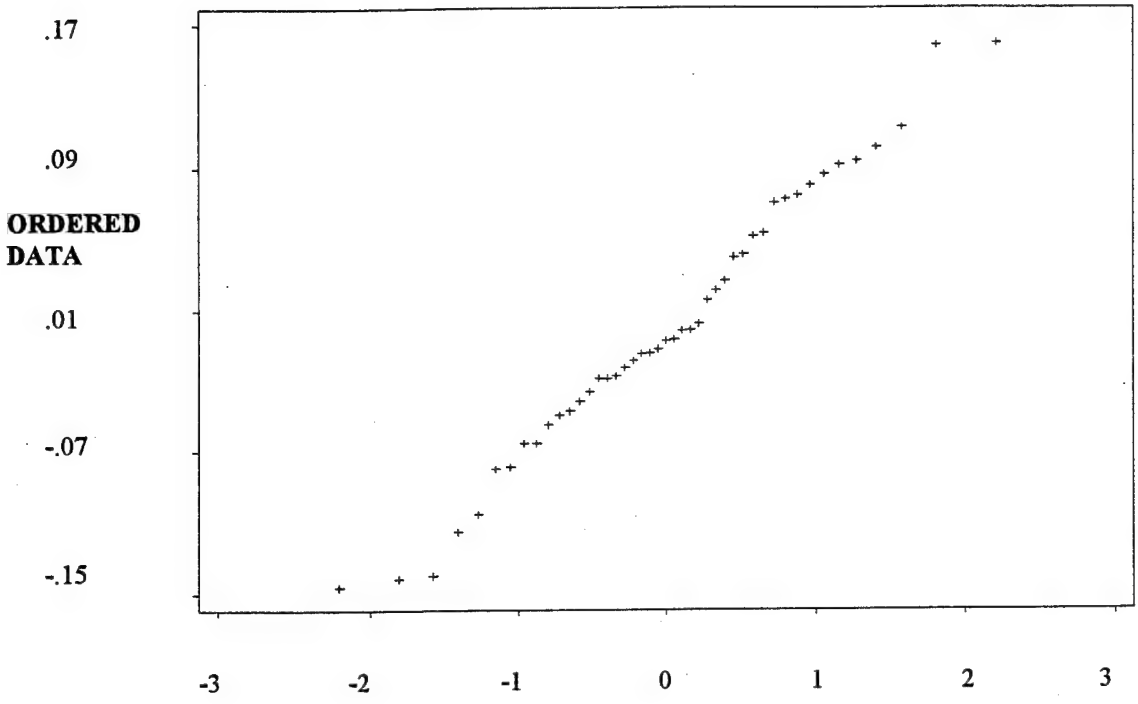


Figure 3-26

Rankit Plot of Residuals

The Tukey comparison was conduct at an 85% confidence level. It used the MSE value to compute the half width confidence interval.

$$Q_{\alpha,I,I,I\cdot(J-1)} \cdot \sqrt{\frac{MSE}{2} \cdot \left(\frac{1}{k} + \frac{1}{k}\right)} \qquad Q_{\alpha,I,I,I\cdot(J-1)} = 3.92$$

MSE=.00838 k=3 observations

If the difference in means is more then .103 then they are significantly different.

The means for each treatment are then lined up in ascending order and the significant differences determined Figure 3-27. The table represents the results of

three Tukey analysis for statistical differences in metal toxicity at a constant pH. Tukey analysis must be repeated for all metals at each of the three pH's.

pH 5	Cu (.486)	Fe (.528)	Zn (.825)	Co (.839)	Mn (1.043)
pH 7	Cu(.201)	Zn(.517)	Co(.7317)	Fe(.932)	Mn(.971)
pH 9	Cu (.368)	Co(.715)	Zn(.848)	Mn(.971)	Fe(1.02)

Figure 3-27

Tukey Multiple Comparison Results for Metals at a Constant pH

Underlined treatment levels are not statistically significantly different

4.0 Results and Discussion

The experiments for this thesis were conducted according to the methodology section in Chapter 3 between 1 May 95 and 5 Sep 95. This chapter will present and analyze the results of this experimentation.

4.1 Metabolic Inhibition of Metals and the Effects of pH

The first experiment to be discussed involves the comparison of normalized metabolic rates for several metals over three pHs. This information will be used to determine the relative metabolic inhibition of the metals, Section 4.1.1 and the effect of pH on inhibition, Section 4.1.2. Corroborating evidence for the pH inhibition will be introduced from the x-ray fluorescence experiments.

Table 4-1
Means and Standard deviations of Normalized Metabolic Activity as a Function of pH
Examining the effect of changing pH on the metabolic inhibition caused by the addition of selected metals (at 4 ppm)

	pH5 mean	pH5 Std	pH7 mean	pH7 Std	pH9 mean	pH9 Std
Cu	.486	.215	.201	.039	.3680	.195
Co	.839	.150	.7317	.071	.715	.154
Fe	.528	.0927	.932	.113	1.019	.011
Mn	1.043	.0212	.9707	.098	.9707	.104
Zn	.8253	.179	.5167	.051	.8477	.172

A two way ANOVA of the experiment was conducted; it was determined that at a α=.05 significance level there was interaction between the metal and the pH. A Wilk-Shapiro plot was constructed for the residuals and yielded a Wilk-Shapiro statistic of .99.

The family-wise MSE (MSE=.00838) was taken from the two way ANOVA and used to compute the difference between means necessary to call them significant at α=.15. This was determined to be .101. If the means of any two comparisons is greater than .101 then they may be declared significantly different. The results of the Tukey method are shown in Figure 4-1.

pH 5	Cu (.486)	Fe (.528)	Zn (.825)	Co (.839)	Mn (1.043)
pH 7	Cu(.201)	Zn(.517)	Co(.7317)	Fe(.932)	Mn(.971)
pH 9	Cu (.368)	Co(.715)	Zn(.848)	Mn(.971)	Fe(1.02)

Cu	pH7 (.201)	pH9 (.368)	pH5 (.486)
Fe	pH 5 (.528)	pH 7 (.932)	pH 9 (1.02)
Zn	pH7 (.517)	pH 5 (.825)	pH 9 (.848)
Co	pH 9 (.715)	pH 7 (.732)	pH 5 (.839)
Mn	pH 7 (.971)	pH 9 (.971)	pH 5 (1.043)

Figure 4-1
Statistical Results of Tukey Analysis
Underlined values are not statistically significantly different

4.1.1 Metal Inhibitory Effects

The first inhibition result considered is the normalized metabolic rate for different metals; they were rank ordered according to their metabolic inhibition. The metals were rank ordered for each pH level. Because of metal phosphate precipitation at higher pHs, pH 5 was chosen as the best indicator of metal toxicity. This inhibition ranking may be compared to other characteristics of the metal that might be used to predict inhibition Table 4-2. The results rank ordered are: pH=5: Cu>Fe>Zn≈Co>Mn; pH=7: Cu>Zn>Co>Fe>Mn; pH=9: Cu>Co>Zn>Mn>Fe.

Table 4-2
Comparison of Inhibition with Two Possible Models of Toxicity
Two methods of characterizing metals were discussed in the literature review; they correspond to microbial toxicity. This chart compares experimental results to these characterization methods

pH 5 Inhibition	Cu>Fe>Zn≈Co>Mn
B class characteristic	Cu>Co≈Fe>Zn>Mn
Ligand preference	Cu>Co>Zn>Fe>Mn
Binding preference with hydrogen phosphate	Cu>Fe>Zn>Co>Mn

The methods of metal characterization discussed in the literature, while adequately describing the inhibition of copper and manganese, did not correctly predict the inhibition of cobalt, iron, and zinc. However, when metabolic inhibition was compared against the metal binding preference of the metal with hydrogen phosphate

there was an exact match. This indicates that a metals ligand binding preference with hydrogen phosphate could be used in the absence of experimental data to predict a metals inhibition compared to other metals.

4.1.2 pH Effects on Cellular Inhibition

A review of the data from this experiment shows that with the exception of iron there is an increase in cellular inhibition for the metals as the pH is raised from pH 5 to pH 7 (Figure 4-2). The most significant differences occur with copper and zinc. The increase in inhibition with increasing pH is predicted from the metal exchange model presented in Section 2.3.2.1. However, the decrease in toxicity between pH 7 and pH 9 is less obvious. An attempt will be made to explain this phenomena as well as to justify the change in toxicity between pH 5 and pH 7.

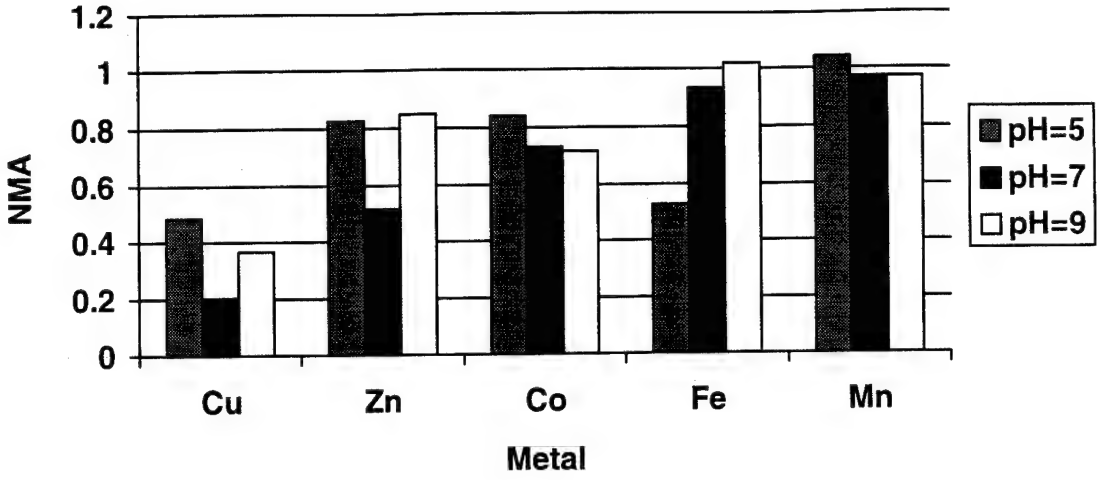


Figure 4-2
Normalized Metabolic Activity as a Function of pH
All Metals are at 4 ppm
Inhibition increases with pH for Cu, Zn, Mn, and Co. Decrease in inhibition between pH 7 and 9 due to precipitation

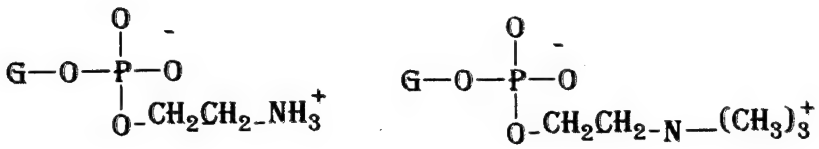
When altering pH the first thing that would be expected to cause a toxicity change would be the presence of OH^- ions or H^+ ions. Several researchers have cited a competition of the metal with hydrogen as the cause for a reduction in toxicity as pH is lowered (Babich and Stotzky, 1980:110); however, a change in the speculation of metal and ligands could also effect microbial inhibition. A simple model of this phenomena is as follows..

$$[M^{n+}]+[Cell] \underset{\longleftarrow}{\overset{K}{\longrightarrow}} [M^{n+}cell] \qquad \text{Toxic} \qquad (4.1)$$

$$[H^{+}]+[Cell] \underset{\longleftarrow}{\overset{K_a}{\longrightarrow}} [Hcell] \qquad \text{Non-toxic} \qquad (4.2)$$

As H^+ increases, equation 4-2 is driven to the right reducing the concentration of [cell] this in turn drives equation 4-1 to the left reducing [M^{N+}Cell]. There is effectively a competition between the hydrogen and metal ions for bonding sites on the cell membrane. Increasing the concentration of hydrogen ions in solution, allows the hydrogen ions to compete better with the metals and acts to lower the amount of metal on the cell. This model depends on many things including the free metal concentration and the type of cellular membrane ligand used in the metal binding.

Although there are many anionic ligands in a cell membrane it is believed that phosphoral groups are the primary sites for metal ligand interactions (Beveridge, 1989, 157). This is primarily because of primary role in the hydrophillic section of the cell membrane. Most often cell membrane phosphate exists as ethanolamine phosphoglyceride or choline phosphoglyceride (Figure 4-3). These esters are highly acidic and exist mostly in the acidic form (Morrison, 1987:1274) vivo; this makes them similar to hydrogen phosphate in their binding potential with metals. To simplify the model phosphate will be selected as the primary binding ligand and all others will be ignored. Having chosen a cellular binding site the exchange model may be revisited using zinc as an example metal.



Ethanolamine phosphoglyceride Choline phosphoglyceride

Figure 4-3
Common Forms of Phosphate in Cell Membranes
Similar to Hydrogen Phosphate. Substitute with hydrogen phosphate for modeling
(Moorison, 1987:1274)

$$[Zn^{2+}]+[HPO_4{}^{2-}] \underset{\longleftarrow}{\overset{10^{15.7}}{\longrightarrow}} [ZnHPO_4] \qquad \text{with } HPO_4 \approx HCell \qquad (4.3)$$

$$[H^+]+[HPO_4{}^{3-}] \underset{\longleftarrow}{\overset{10^{19.5}}{\longrightarrow}} [H_2PO_4{}^{3-}] \qquad (4.4)$$

Subtracting Eq 4-4 from Eq 4-3 yields:

$$[Zn^{2+}]+[H_2PO_4{}^{3-}] \underset{\longleftarrow}{\overset{10^{-3.8}}{\longrightarrow}} [H^+]+[ZnHPO_4{}^{1-}] \qquad (4.5)$$

The equilibrium concentration equation becomes:

$$\frac{[ZnHPO_4{}^{1-}]}{[HPO_4{}^{3-}]} = 10^{-3.8} \cdot \frac{[Zn^{2+}]}{[H^+]} \approx \frac{[ZnCell]}{[HCell]} \qquad (4.6)$$

The model ignores zinc species other than free metal zinc ions; thus it is still requiring the free metal concentration of zinc. The speciation of zinc is effected not only by the pH but also other ligands. A look at other possible ligands in the growth solution reveals that phosphate available as PO_4^{3-}, HPO_4^{2-}, or $H_2PO_4^{1-}$ might in addition to OH^- act

to reduce the concentration of free zinc in solution and could possibly act to reduce the availability and species of metals to the microorganisms. The form of phosphate is also effected by pH; a chart of the speciation of phosphate as a function of pH is given in Figure 4-4. Chlorides, and nitrates were also available as anionic ligands; however these were ignored because of their relatively low binding strengths as compared to phosphate.

It becomes a competition between the cell membranes and aquatic phosphate species for the free metals. Because the metals have an inhibitory effect on the microorganisms, it can be assumed that the ligand binding constant with aquatic phosphate is not significantly greater than the cell's association constant. In addition, an increase in phosphate nutrients (Section 4.5) has an effect on the metal's inhibition; hence, the cell's ligand sites do not have a significantly greater metal association constant than the aquatic phosphate.

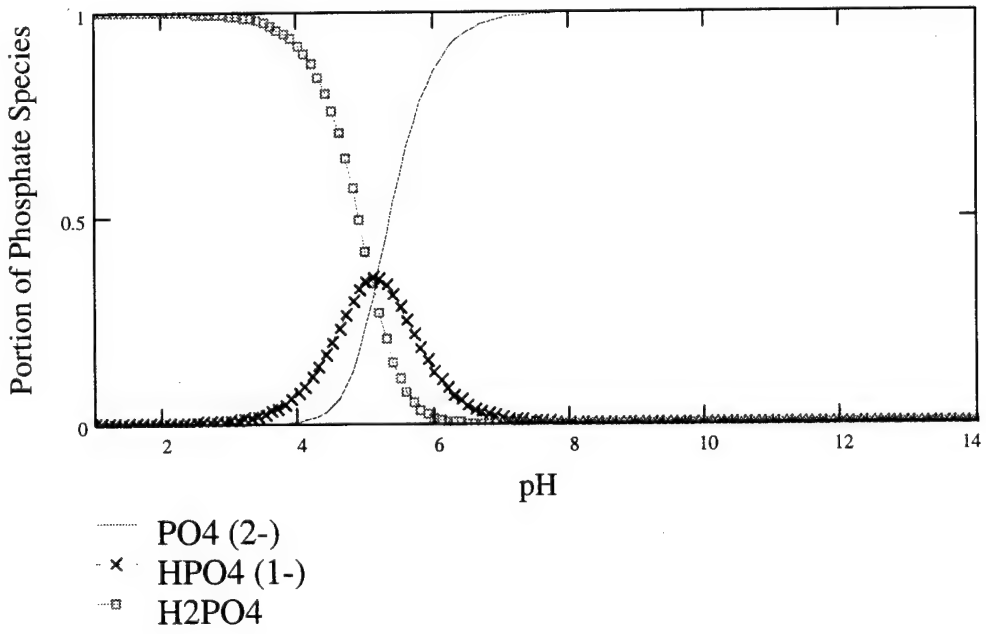


Figure 4-4
Phosphate Speciation as a Function of pH

A Speciation models zinc (Figure 4-5) was constructed using the ligand association constants found in Table 2-2.

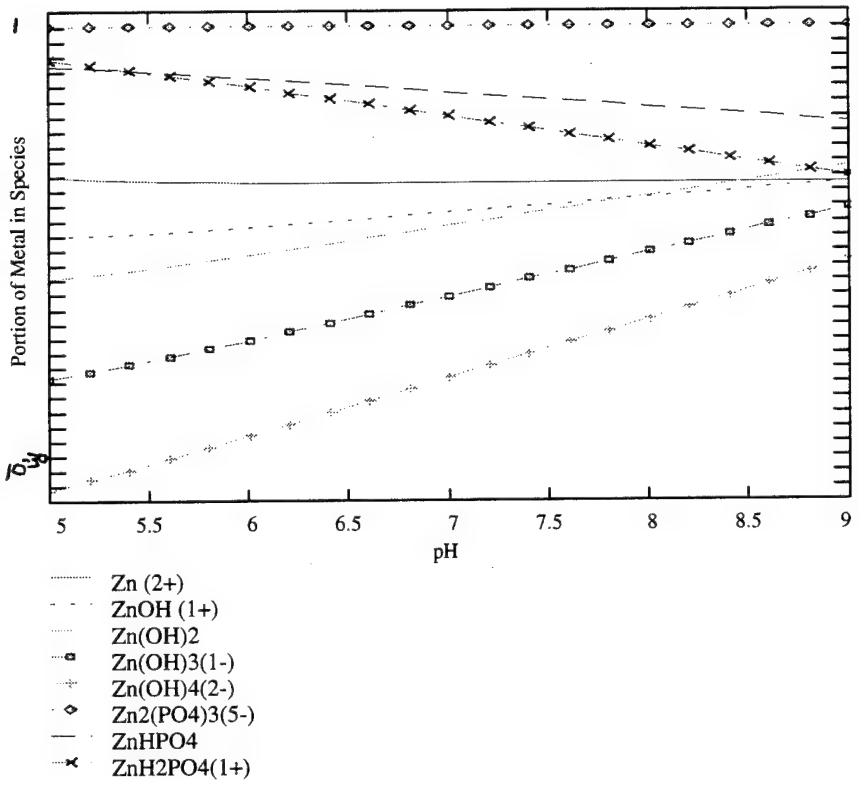


Figure 4-5
Speciation of Zinc
Free zinc remains at a very low concentration through the entire rang of pH values. Zinc phosphate complexes dominate the microcosm

The results show that at the pH's tested, metals will primarily exist as a phosphate ligand complex. Free metal concentrations of zinc was modeled to be:

pH 5 Zn=3.32×10^{-11} thus $\frac{[ZnPO_4^{1-}]}{[HPO_4^{3-}]} = 2.5 \cdot 10^{-10}$

pH 7 Zn=3.35×10^{-11} thus $\frac{[ZnPO_4^{1-}]}{[HPO_4^{3-}]} = 2.5 \cdot 10^{-12}$

This simplistic model would explain the increased inhibition with increased pH. However it is not the only possible explanation for the varying toxicity. The model predicts increasing amounts of ZnOH and $Zn(OH)_2$ in the solution. These could still be toxic to microorganisms. In addition, the presence of zinc phosphate complexes may also have unique toxicities that would effect the outcome of the experiments.

Repeating the experiment at 16 ppm metals concentration (Table 4-3) yields the same pattern of results as 4 ppm as pH is increased and could also be explained by the same model.

Table 4-3
Normalized Metabolic Activity of Metals at 16 ppm as a Function of pH

	pH 5	pH 7	pH 9
Cu	.375	.212	.337
Co	.895	.591	.887
Fe	.317	.695	1.110
Mn	.895	.982	1.187
Zn	.841	.287	.922

The metal exchange model correctly predicts the increased toxicity of a metal as pH changes from 5 to 7; however, it does not predict the decrease in toxicity from 7 to 9. To answer this question results from the x-ray fluorescence experiment must be introduced (Table 4-4).

Table 4-4

Mean and Standard Deviations of Metal/Phosphorous Ratios with Changing pH

The higher the ratio, the more metal there is on the microorganism. Values over 1 have linked to precipitation of the metal from solution through changes in sample weight and x-ray spectra

	Cu/P mean	Cu/P std	Co/P mean	Co/P std	Zn/P mean	Zn/P std
pH5	.927	.0262	.213	.00194	.188	.0104
pH7	1.609	.178	.247	.00990	1.299	.0164
pH9	1.704	.117	1.837	.0671	1.443	.00592

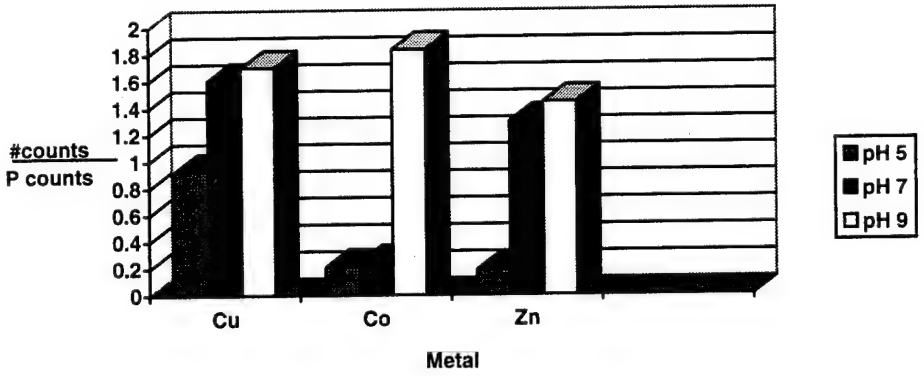


Figure 4-6

M/P for Cu, Co and Zn as a Function of pH

The change between Co pH 5 and 7 are the only two reading in which a comparison can be made without precipitation influences

In theory the quantity of metal counts is proportional to the quantity of metal in the sample while the quantity of phosphorous counts should be proportional to the quantity of microorganisms in the sample. The ratio of metal counts to phosphorous counts (M/P) represents the amount of metal on a microorganism. As pH increases we would expect to see the ratio of M/P counts increase thus representing an increase in

metals on the microorganisms. The problem is that when metals precipitate out of solution the assumptions are no longer valid. This has occurred in most instances in this XRF experiment. The lone exception is with cobalt between pH 5 and pH 7. Here a small but significant increase occurs; this is consistent with an increase in adsorption to a cell membrane. Out of the entire experiment this is the lone example of a correlation between toxicity and adsorption with varying pH.

The normalized metabolic activity of a microcosm dosed with cobalt at pH 7 was 86.9±2.66% of that at pH 5; while the quantity of Co/P at pH 5 was 87.5±0.5% of that at pH 7. This is a very good correlation between toxicity and adsorption.

In the remainder of the results the metal dropped out of solution as some sort of phosphate hydroxide complex. The results of the precipitation can be seen several ways. First the filters may take on the color of the precipitate; the best example of this was with cobalt. At pH 5 and 7 the filter paper was brown due to the microorganisms; however at pH 9 a lavender precipitate was observed. Secondly precipitation may be seen with the XRF. As precipitate occurs M/P values increase dramatically (eg. from .247 for cobalt at pH 7 to 1.837 at pH 9) and the number of counts for both the metal and phosphorous increase drastically (~23,000 Co and ~100,000 P for Co at pH 7 to ~1,300,000 Co and ~700,000 P at pH 9). The best way to see this is by observing the differences in x-ray spectra (Figures 4-7 through 4-10); note the change in height of the phosphorous (P) and cobalt (Co) peak compared to rhodium (Rh). The rhodium peak is a reflection of the rhodium target x-rays off of the filter paper; its total counts is independent of any elements on the sample. A third way to determine precipitation is to compare the masses

of the filters. If the filter weight increases dramatically with pH then precipitation is occurring.

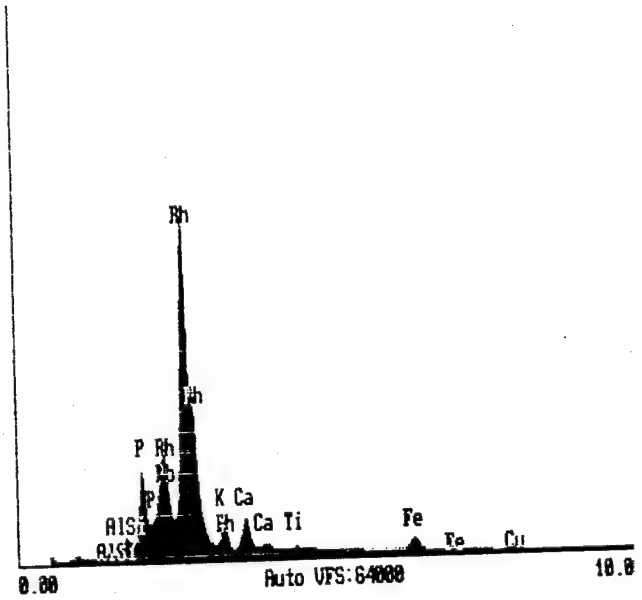


Figure 4-7
Spectrum of control
Note the height of phosphorous (P) to rhodium (Rh); the rhodium is constant throughout all samples because it is the result of reflection of the target material off the sample

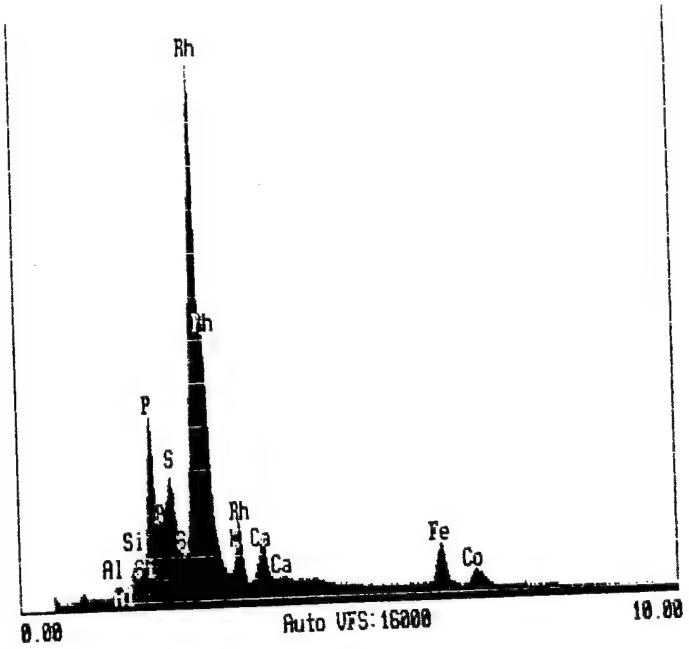


Figure 4-8
Spectrum of Microorganisms dosed with cobalt at pH 5
Note the height of phosphorous (P) to rhodium (Rh); the rhodium is constant throughout all samples because it is the result of reflection of the target material off the sample

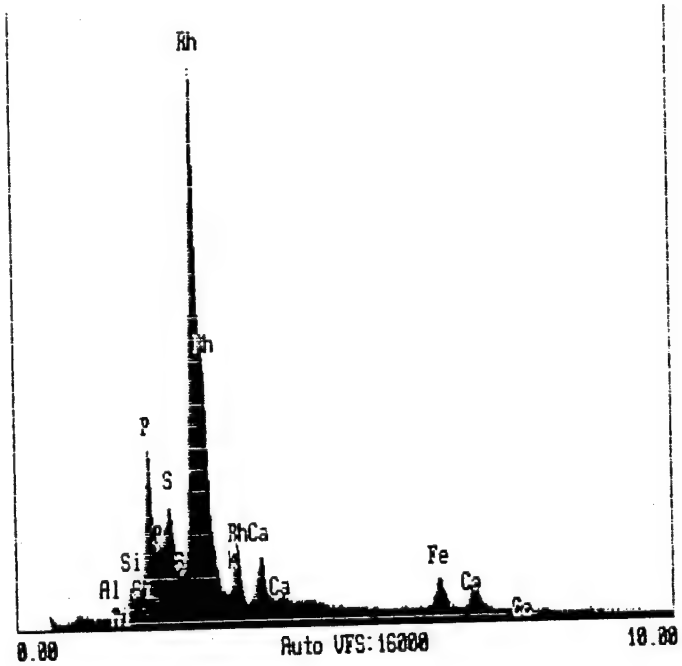


Figure 4-9
Spectrum of Microorganisms dosed with cobalt at pH 7
Note the height of phosphorous (P) to rhodium (Rh); the rhodium is constant throughout all samples because it is the result of reflection of the target material off the sample

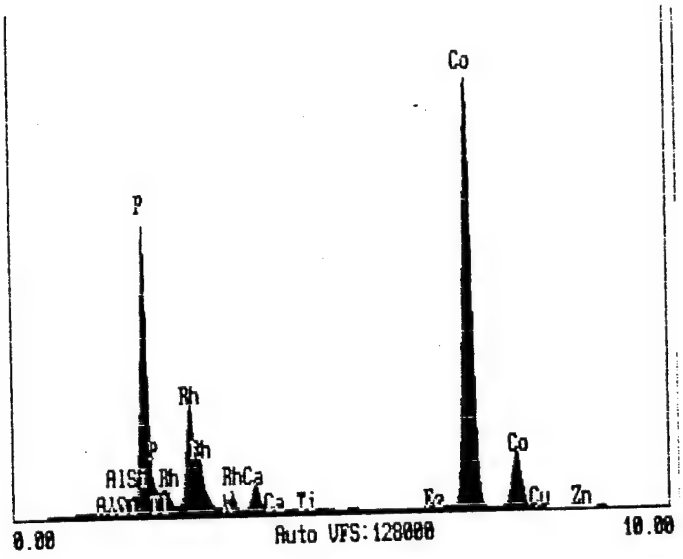


Figure 4-10
Spectrum of Microorganisms dosed with cobalt at pH 9
Note the height of phosphorous (P) to rhodium (Rh); the rhodium is constant throughout all samples because it is the result of reflection of the target material off the sample. At this pH the number of phosphorous and cobalt counts increases dramatically as compared to the constant rhodium peak. This increase is accompanied with an increase in weight and color of the filter paper

The precipitation of metals explains the decrease in inhibition between pH 7 and pH 9, as the pH increases the equilibrium between solid and aqueous phase in equation 4-7 is driven left. [OH] is increased by two orders of magnitude, PO_4^{3-} is so much larger than the other concentrations that it is virtually unaffected in concentration. This leaves the aqueous metal concentration [M] which will decrease. The lower concentration of [M] means less is available to the cell for adsorption; thus a reduction in toxicity should and does occur.

$$a[M]+b[PO_4]+c[OH^-] \underset{\longleftarrow}{\overset{K_{sp}}{\longrightarrow}} [M_a(PO_4)_b(OH)_c] \qquad (4.7)$$

4.2 Effects of Magnesium on the Metabolic Inhibition by Metals

The second experiment to be discussed involves the addition of a competitive cation of lesser toxicity to a microcosm treated with another metal. The metal under investigation are zinc, copper, and cobalt and the competitive cation is magnesium. According to literature review, Section 2.5.4, one can expect to see a decrease in toxicity, an increase in toxicity or no change in toxicity at all as the concentration of competitive cations are increased. This experiment explores the effect of magnesium on metal inhibition. The results of the experiment can be seen in Table 4-5 and Figure 4-12.

Table 4-5
Normalized metabolic activity at 8 ppm Metal addition
as it is affected varying levels of Mg^{2+}
Notice that as magnesium levels are increasing the inhibition of the metal is decreasing. This indicates that the magnesium is abating the toxicity of the metal. The experiment is performed at pH 7

Mg^{2+}	Cu mean	Cu std	Co mean	Co std	Zn mean	Zn std
0 ppm	.255	.010	.289	.014	.500	.019
50 ppm	.261	.028	.614	.052	.629	.065
100 ppm	.289	.005	.892	.024	.705	.018

A two way ANOVA of the experiment was conducted. It was determined that there was interaction between the metal and magnesium level at the α=.05 significance level. A Wilk-Shapiro plot was constructed for the residuals and yielded a Wilk-Shapiro statistic of .95.

The family wise MSE ($MSE=5.182x10^{-4}$) was taken from the two way ANOVA and used to compute by the Tukey analysis the difference between means necessary to

call them significant at α=.15. This was determined to be .0289. If the means of any two comparisons is greater than .0289 then they may be declared significantly different. The results of the Tukey method are presented below (Figure 4-11).

Cu	0 ppm (.255)	50 ppm (.261)	100 ppm (.289)
Co	0 ppm (.289)	50 ppm (.614)	100 ppm (.892)
Zn	0 ppm (.500)	50 ppm (.629)	100 ppm (.705)

Figure 4-11
Tukey Results for Effect of Magnesium Experiment

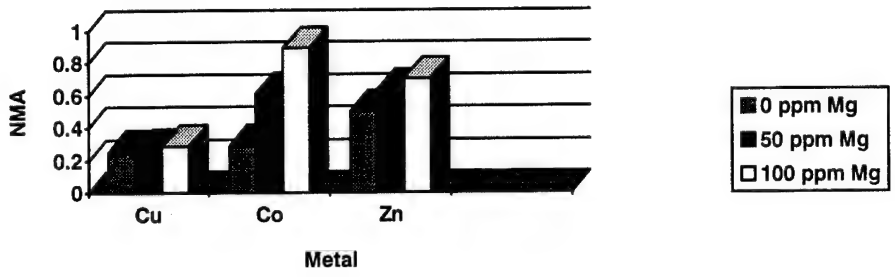


Figure 4-12
Normalized Metabolic Activity as a Function of Magnesium concentration
Note the decrease in inhibition as the concentration of magnesium is increased

The results of the experiment reveal that the normalized metabolic activity rate increases with increasing concentration of Mg^{2+}. This indicates that Mg^{2+} may be competing with metals for bonding sites on the cell surface. Using the model for toxicity built in Section 4.1.1 and substituting Mg^{2+} for H^{+} a new model is created. The model shows that as $[Mg^{2+}]$ is increased the concentration of metal on the cell [Mcell] decreases.

$$[Mg^{2+}]+[Cell] \underset{\leftarrow}{\overset{K}{\rightarrow}} [Mg \cdot cell] \text{ (not toxic)} \tag{4.8}$$

$$[M^{2+}]+[Cell] \underset{\leftarrow}{\overset{K'}{\rightarrow}} [M \cdot cell] \text{ (toxic)} \tag{4.9}$$

$$\frac{M \cdot cell}{Mg \cdot cell} = \frac{[M]}{[Mg]} \cdot 10^{K'-K} \tag{4.10}$$

The results of the XRF experiment with magnesium shows that the M/P ratio of cobalt and zinc does indeed decrease with increasing Mg. The results may be seen in Table 4-6. In this experiment copper had precipitated and therefore its results were not interpretable; zinc and cobalt had good correlation. A plot of NMA, Mg/P, and P/M (inhibition, magnesium adsorbed onto the microorganism, and the inverse of metal adsorbed to microorganism respectively) vs magnesium concentration, for zinc, (Figure 4-13) indicates a general trend as magnesium is increased. This would indicate that as the magnesium concentration is increased more and more zinc is displaced from the cell membrane and this causes inhibition to decrease.

Table 4-6
Results of X-ray Spectroscopy experiment on the effect of Mg^{2+} at pH 7 on adsorption

Metal	Treatment	Mg/P mean	Mg/P Std	M/P mean	M/P Std
	0 ppm	-	-	.840	-
Cu	50 ppm	.0644	.00240	1.246	.0469
	100 ppm	.0663	.00287	1.262	.0156
	0 ppm	-	-	.1420	.0148
Co	50 ppm	.0943	.000294	.1103	.00572
	100 ppm	.0964	.000616	-	-
	0 ppm	-	-	.6363	.137
Zn	50 ppm	.0746	.00157	.5127	.0411
	100 ppm	.0964	.0168	.3087	.0605

A comparison of normalized metabolic rates with XRF results Table 4-7 shows a relationship between NMA and magnesium and an inverse relationship to the metal adsorbed. NMA increases with increasing Mg level, the level of metal (zinc or cobalt)decreases with increasing Mg level and the amount of Mg increases with increasing Mg level.

Table 4-7
Comparison of NMA, M/P and Mg/P as the Magnesium Concentration is Increased

Metal	treatment	NMA	M/P	Mg/P
	0 ppm	.500	.636	-
Zn	50 ppm	.629	.513	.0746
	100 ppm	.705	.309	.0964
	0 ppm	.289	.142	-
Co	50 ppm	.614	.113	.0746
	100 ppm	.892	-	.0964

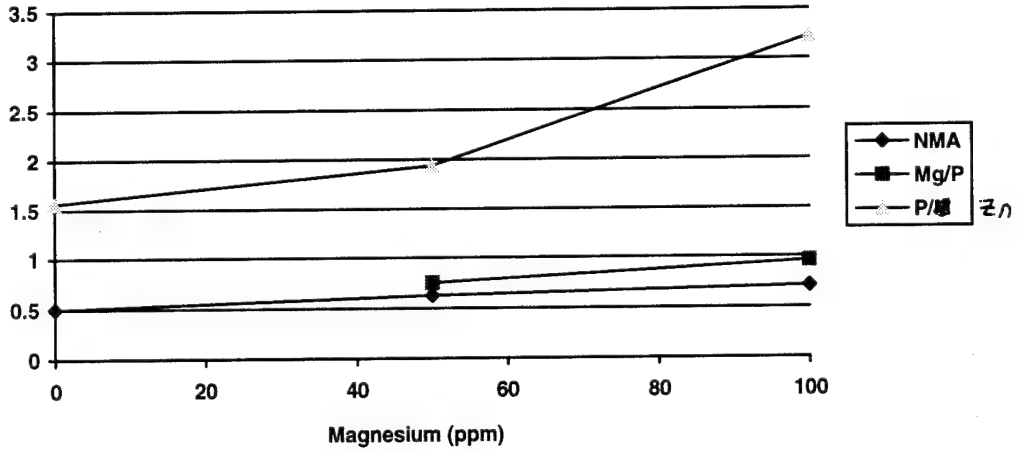


Figure 4- 13
NMA, 10*Mg/P, and the Inverse of Zn/P for Varying Concentrations of Magnesium

If the cell surface is modeled as a phosphate ion HPO_4^{3-} the ligand association constants show that copper is most tightly bound $p[CuHPO_4]=16.5$, followed by zinc, $p[ZnHPO_4]=15.7$, and then cobalt, $p[CoHPO_4]=15.5$. This would suggest that Magnesium competing with the metals for cell binding sites $[MgHPO_4]=15.3$] would compete most successfully with cobalt and have the greatest reduction in toxicity with this metal. The second biggest reduction in toxicity would result from competition with zinc, followed by copper. This is indeed the case see Table 4-8.

Table 4-8
Change in NMA as a Result of Increasing Mg^{2+} Concentrations
The magnitude of change in inhibition corresponds to the order of decreasing ligand association constants

	Cu ΔNMA	Zn ΔNMA	Co ΔNMA
0-50 ppm	.006	.129	.325
50-100 ppm	.028	.076	.278
Log (K)	16.5	15.7	15.5

4.3 Effect of Calcium on the Metabolic Inhibition by Metals

The third experiment to be discussed is a repeat of experiment #2 however this time Ca^{2+} was used as the competitive cation instead of magnesium. The results of this experiment are presented in Table 4-9 and Figure 4-14.

Table 4-9
Normalized Metabolic Activity at 8 ppm
as it is Effected by Varying Levels of Ca^{2+}
The inhibition of metals decreases as Ca is increased from 0 to 50 ppm but the inhibition increases as calcium is increased from 50 to 100 ppm for cobalt and zinc

	Cu mean	Cu std	Co mean	Co std	Zn mean	Zn std
0	.201	$7.3x10^{-4}$	.620	$1.6x10^{-3}$	.507	$9.2x10^{-4}$
50	.255	$2.9x10^{-3}$	.863	.0246	.757	$1.6x10^{-3}$
100	.294	$2.9x10^{-3}$	.6917	$5.9x10^{-4}$	.700	$6.3x10^{-4}$

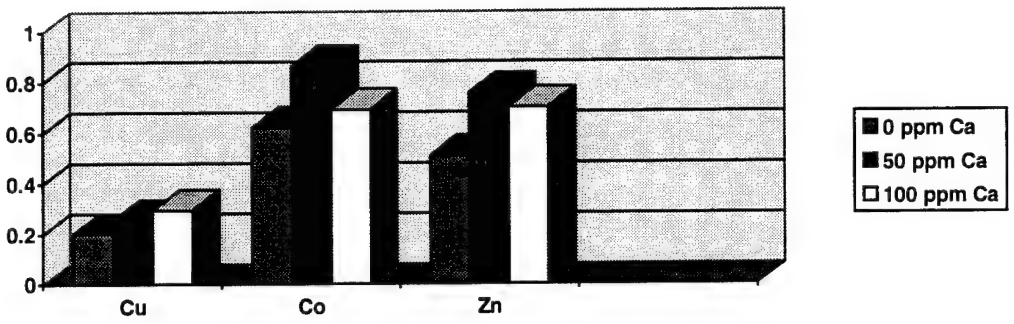


Figure 4-14
Normalized Metabolic Activity as a Function of Calcium Concentration
The inhibition of metals decreases as Ca is increased from 0 to 50 ppm but the inhibition increases as calcium is increased from 50 to 100 ppm for cobalt and zinc

A two way ANOVA of the experiment was conducted. It was determined that there was interaction between the metal and calcium level at α=.05 significance level. A Wilk-Shapiro plot was constructed for the residuals and yielded a Wilk-Shapiro statistic of .81.

The family wise MSE (MSE=.00174) was taken from the two way ANOVA and used to compute the difference between means necessary to call them significant at α=.15. This was determined to be .0497. That is, if the difference of the means of any two comparisons is greater than .0497 then they may be declared significantly different. The results of the Tukey comparison are presented below (Figure 4-15).

Cu	0 ppm (.201)	50 ppm (.255)	100 ppm (.294)
Co	0 ppm (.620)	100 ppm (.692)	50 ppm (.863)
Zn	0 ppm (.507)	100 ppm (.700)	50 ppm (.757)

Figure 4-15
Results of Tukey Analysis for the Effect of Calcium on Inhibition

The results of this experiment were expected to be the same as those of magnesium; however, there were very different. Instead of reducing the toxicity of metals, as calcium concentration increased in two cases, cobalt and zinc, toxicity decreased initially only to increase again as calcium concentration continued to rise. It is possible that calcium acts competitively with bonding sites on the cell membrane; however, after a certain concentration Ca begins to successfully compete with Zn and Co for phosphate ligands in the dilution water this increases the concentration of Zn^{2+} and Co^{2+} in the solution. The increased free metal concentration would increase lead to increased toxicity. If this explanation is correct then Ca would have a greater effect on the increased toxicity of cobalt than zinc. This is a result of the fact that the association constant of $p[CoHPO_4]$= 15.5 is less than zinc, $p[ZnHPO_4]$= 15.7, both of which are close

to Calcium p[$CaHPO_4$]= 15.1. Copper association constant p[$CuHPO_4$]= 16.1 is a full order of magnitude higher than calcium and thus not as easily replaced.

If this explanation holds true then we would expect to see the largest changes in toxicity between varying levels of cobalt, followed by zinc and finally copper. Table 4-10 explores this theory, with the exception of cobalt and zinc between 0 and 50 ppm the correlation holds.

The relationship to adsorption was not studied because time restrictions did not allow for XRF analysis of the metal adsorption in this experiment. Had x-ray analysis been preformed a difference in adsorption may have been discovered that might have explained the inhibition results.

Table 4-10

Change in NMA as a result of increasing Ca^{2+} Concentrations

	Cu ΔNMA	Co ΔNMA	Zn ΔNMA
0-50 ppm	.054	.243	.257
50-100 ppm	.039	-.171	-.057

4.4 Effect of Population Size on the Metabolic Inhibition by Metals

The effect of population size on the toxicity of a metal was tested in the next experiment. In this experiment varying levels of microorganism innoculum were tested on three metals copper, cobalt, and zinc. These results are presented in Table 4-11 and Figure 4-16.

Table 4-11
The Effect of Changing Microorganism Population Size on Normalized Metabolic Activity

Note that inhibition for zinc is increased as population size is increased. This is as of yet unexplained.

	Cu mean	Cu std	Co mean	Co std	Zn mean	Zn std
10 ml innoc	.068	.00714	.909	.0469	.410	.00520
30 ml innoc	.073	.00412	.933	.0313	.321	.0346
50 ml innoc	.085	.0207	.952	.0490	.239	.0241

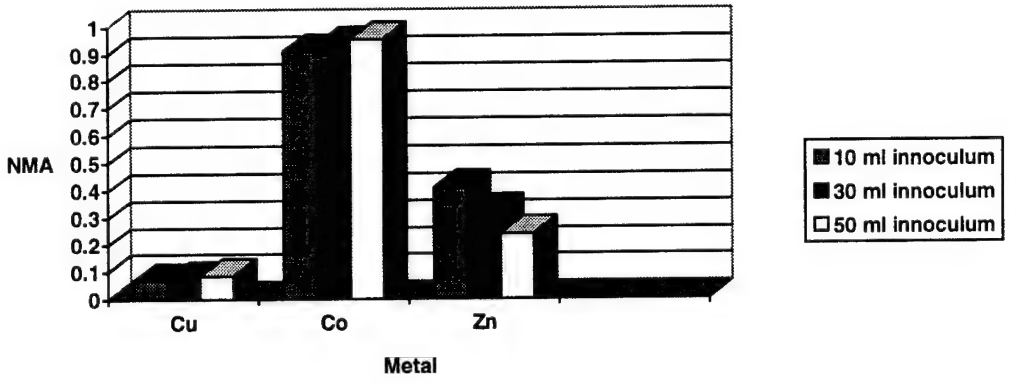


Figure 4-16
Effect of Microbial Population on NMA
For Cu 16 ppm, Co and Zn 33 ppm

As microbial population is increased the amount of inhibition caused by metals was determined. It showed that the normalized metabolic activity for copper and cobalt remained virtually unchanged as population size was increased; however, zinc showed increasing inhibition as population size is increased.

The experiment was conducted at pH 7.

A two way ANOVA of the experiment was conducted. It was determined that there was no interaction between the metal and population size at α=.05 significance

level. A Wilk-Shapiro plot was constructed for the residuals and yielded a Wilk-Shapiro statistic of .81.

The family wise MSE (MSE=4.395×10^{-4}) was taken from the two way ANOVA and used to compute the difference between means necessary to call them significant at α=.15. This was determined to be .0251. That is if the means of any two comparisons is greater than .0251 then they may be declared significantly different. The results of the Tukey comparison are presented in Figure 4-17.

Cu	10 ml (.068)	30 ml (.073)	50 ml (.085)
Co	10 ml (.909)	30 ml (.933)	50 ml (.952)
Zn	50 ml (.239)	30 ml (.321)	10 ml (.410)

Figure 4-17
Results of Tukey Analysis for the Effect of Population Size on Metabolic Inhibition

The results of this experiment may be explained by returning to our original model of cellular toxicity and adding two equations.

$$[M^{n+}]+[Cell] \underset{\longleftarrow}{\overset{K}{\longrightarrow}} [M^{n+}cell] \tag{4.1}$$

$$[H^{+}]+[Cell] \underset{\longleftarrow}{\overset{K_a}{\longrightarrow}} [Hcell] \tag{4.2}$$

$$[Cell_{total}]=[Cell]+[Hcell]+[Mcell] \tag{4.11}$$

$$[M_{tot}]=[M]+[Mcell]+[MHPO_4]+... \tag{4.12}$$

As cell totals are increased the quantity of [Mcell] will increase as will the quantity of [Hcell] and [cell]. If inhibition is equated to the ratio $\frac{[Mcell]}{[Hcell]}$, how this inhibition ratio changes with increasing population size will depend on the initial quantity of metal in solution and how well the cell site competes with ligands in solution for the metal. If the total amount of metal was high compared to the cell population and the cell competed well with the ligands in solution then one would expect the [M] concentration to remain relatively constant as [$Cell_{tot}$] increased thus $10^{K}.\frac{[Zn^{2+}]}{[H^{+}]}$ would remain constant and inhibition would remain relatively constant.

If [Mcell] did not compete well with ligands in the microcosm or [M_{tot}] was small compared to [$Cell_{tot}$] then [Zn^{2+}] would decrease as the total quantity of cells increased and $10^{K}.\frac{[Zn^{2+}]}{[H^{+}]}$ would decrease thus lowering inhibition.

This would explain the increase in NMA as population size increased for copper and cobalt. It would not explain zinc's decrease in NMA with increasing population size. As of yet no possible explanation has been postulated for this result.

4.5 Effect of Nutrient Levels on the Metabolic Inhibition by Metals

In this experiment the effect of nutrients on the normalized metabolic rate of metal dosed microcosms was investigated. The experiment's purpose was to demonstrate correlation between the level of inorganic nutrients (i.e. competitive binding ligands) and toxicity. The results are presented in Table 4-12.

Table 4-12

Effect of Changing the Nutrition Level on the Normalized Metabolic Activity

Metal inhibition was examined in changing nutritional environments. As nutrients were increased, the inhibition caused by the metals decreased. The was presumable the result of metal-phosphate bonding. The results did not include the effects of pH changes brought about by the additional nutrients

	Cu mean	Cu std	Co mean	Co std	Zn mean	Zn std
Low	.011	.00412	.630	.331	.436	.0241
Medium	.130	.0181	.605	.0122	.673	.0122
High	.318	.0458	.632	.0373	1.12	.0889

A two way ANOVA of the experiment was conducted. It was determined that there was interaction between the metal and nutrient level at α=.05 significance level. A Wilk-Shapiro plot was constructed for the residuals and yielded a Wilk-Shapiro statistic of .66.

The family wise MSE (MSE=.0066) was taken from the two way ANOVA and used to compute the difference between means necessary to call them significant at α=.15. This was determined to be .094. That is if the means of any two comparisons is greater than .094 then they may be declared significantly different. The results of the Tukey analysis are presented below in Figure 4-18.

Cu	Low (.011)	Med (.130)	High (.318)
Co	Med (.605)	Low (.630)	High(.632)
Zn	Low (.436)	Med (.673)	High (1.12)

Figure 4-18

Results of Tukey Analysis on the Effects of Nutrient Levels on Metabolic Inhibition

The design of this experiment failed to take into account the pH effects of the dissociation of $NH_4H_2PO_4$ to PO_4^{3-}. As a result the pH of the low nutrient level is greater than that of the medium which is greater than those of the high nutrient level. No concrete conclusions can be drawn because as has already been demonstrated pH has an effect on toxicity. It should be noted, however that the normalized metabolic rates for copper and zinc changed substantially between the three levels. The change was much greater than the changes in the pH experiments alone. It is believed that this is a result of the increased concentration of PO_4^{3-} in solution which acts to competitively bind the metal thus protecting the cell.

In addition, the magnitude of NMA increase for each metal as nutrient levels were increased corresponded to magnitude of the metal-phosphate ligand binding indicating increased competition with phosphate ligands causes the microorganism to have less metal available to them for binding (Table 4-13). The lack of availability metal due to competition with other ligands leads to lower inhibition.

Table 4-13
Comparison of Nutrient Effects to Hydrogen Phosphate Ligand Binding Constants

	% change NMA	Log (K)
Cu	2800	16.5
Zn	256	15.7
Co	-	15.5

4.6 Effect of Sodium Chloride on the Metabolic Inhibition by Metals

This experiment was designed to investigate the effects of varying chloride ion levels on the normalized metabolic rate of microcosms dosed with metals. Originally,

this was an attempt to explain the increased toxicity of zinc with increasing innoculum; however, the results (Table 4-14) showed something more interesting

Table 4-14

Normalized Metabolic Activity as it is Effected by Sodium Chloride

	Cu mean	Cu std	Co mean	Co std	Zn mean	Zn std
0 ppm Cl^-	.1993	.0277	.462	.0346	.179	.00911
166 ppm Cl^-	.0670	.0100	.575	.00458	.204	.0288
333 ppm Cl^-	.0643	.0130	.595	.0728	.193	.0207

A two way ANOVA of the experiment was conducted. It was determined that there was interaction between the metal and nutrient level at α=.05 significance level. A Wilk-Shapiro plot was constructed for the residuals and yielded a Wilk-Shapiro statistic of .99.

The family wise MSE (MSE=3.1×10^{-4}) was taken from the two way ANOVA and used to compute the difference between means necessary to call them significant at α=.15. This was determined to be .021. That is if the means of any two comparisons is greater than .021 then they may be declared significantly different. The results of the Tukey analysis are presented below (Figure 4-19)

Cu	0 ppm (.199)	166 ppm (.067)	333 ppm (.064)
Co	0 ppm (462)	166 ppm (.575)	333 ppm (.595)
Zn	0 ppm (.179)	333 ppm (.193)	166 ppm (.204)

Figure 4-19

Results of Tukey Analysis for the Effect of Sodium Chloride on Metabolic Inhibition

The addition of large amounts of chloride to the microcosm should not have significantly altered the equilibrium state of the metals, and thus toxicity. The ligand binding coefficients for the zinc, copper and cobalt with chloride are 0.4, 0.5, and 0.5 respectively; compared to phosphate, 15.7, 16.1, and 15.5 they are insignificant and the hydrogen phosphate ligand association constant with sodium is 13.5, much lower than the other metals. Yet, copper became significantly more toxic as chloride was added and cobalt significantly less toxic. Zinc remained relatively constant in toxicity. This may be an example of an inorganic ligand increasing toxicity with one metal and abating it with another; however further work would have to be done before that statement could be made with any certainty.

4.7 Investigation of the Microbe Mortality by Metals

It was of interest to know if the metals being tested were causing the reduction in normalized metabolic rate by inhibiting the cells respiration or by killing them. This experiment examines the effect of metals on population viability and diversity. The results are presented in Table 4-15.

Table 4-15
Plate Results of Microorganisms dosed with metals at 50 ppm
(number of colonies / number of colony types)

	Control	Cu	Fe	Co	Mn	Zn
M. Endo E. coli	501 9	77 2	31 2	478 9	333 6	355 5
M-TCG General	3264 5	122 3	13 2	2631 3	1837 5	2592 4
M-Green fungi	73 5	0 0	3 2	56 3	64 1	66 1

The population data presented is the result of 1 sample therefore no statistical analysis may be accomplished. In addition, this experiment is highly dependent on the homogeneity of all samples, thus large variations are possible and only general conclusions may be reached.

It is possible to conclude that the innoculum consists of a diverse and very large population. At a 1:100 dilution the microbial population was too numerous to count (TNTC) when grown on the general heterotrophic medium, and had nine colony types when grown in an *E. coli* growth medium. The diversity of colony types does not mean that there are nine different species but instead is indicative of a diverse population. In addition, there was also a healthy population of fungi with five different colony types. It was encouraging for the validity of the results that the number of colony types and species of test samples were always less than the control.

Attempts to correlate the plate count numbers with the metabolic inhibition of the metals tested failed to yield equivalent results.

Plating results: Fe>Cu>Mn>Zn>Co

Metabolic Inhibition: Cu>Fe>Zn≈Co>Mn

Although the iron toxicity may readily be explained by the added toxicity due to the Lewis acid nature of the metal (pH<3, at 50 ppm), there is no obvious explanation for the lack of correlation for the other metals. The difference in results may be accounted for by population variation in the experiment, non-homogeneity during sampling, or that NMA is not a good indicator of cellular mortality.

If the ratio of metal dosed colonies to control colonies is computed and compared to the NMA for the metals at 50 ppm (Table 4-16) some interesting observations can be made.

Table 4-16
Normalized Metabolic Activity vs. Colony Counts
Inhibition and morbidity results did not correlate well. It was able to be concluded that copper was toxic to microbes while zinc was inhibitory

	$\frac{\#colonies}{\#control}$ bacteria	NMA	$\frac{\#colonies}{\#control}$ fungi
Cu	.037	.050	0
Co	.806	.675	.767
Mn	.562	.900	.874
Zn	.794	.220	.904

It would appear from these results that copper is indeed fatal to the microorganisms and that zinc is inhibitory. Because no statistics are available for cobalt no comments can be made as to the lethality or inhibitory nature of this metal. Manganese is interesting; for a metal that does not effect NMA much it appears to be quite lethal. Again these findings are preliminary, based only on one observation, yet are interesting and warrant further study.

4.8 Effect of Varying Metal Concentration on NMA

This experiment explores the effect of changing metal concentrations on the NMA of metal dosed microcosms. The model described in the literature review for percent inhibition can be used to estimate the normalized metabolic rate.

$$\%inhibition = \frac{K \cdot [M]}{1 + K \cdot [M]} \qquad \text{(Morel:1992:412)} \qquad (4.12)$$

$$NMA = \frac{inhibited \cdot respiration}{uninhibited \cdot respiration} = 1 - \%inhibition = 1 - \frac{K \cdot [M]}{1 + K \cdot [M]} \qquad (4.13)$$

Where:

K is the inhibition constant
[M] is the metal concentration

The model predicts a decreasing rate of inhibition increase as the metal concentration increases. The inhibition is expected to increase at a decreasing rate until all metabolic activity ceases, Figure 4-20.

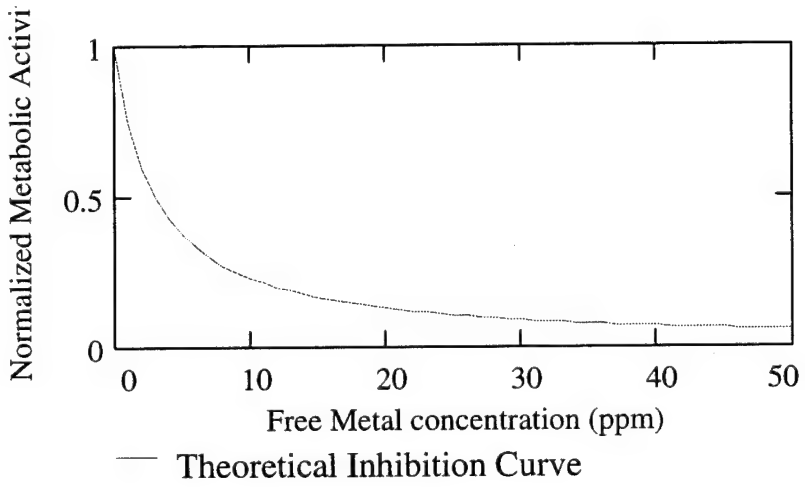


Figure 4-20
Theoretical Inhibition Curve
As the total metal concentration is increasing, the quantity of inhibitory metal continues to increase until all cellular receptors are occupied. This results in maximum inhibition, and most likely death

The results of increasing metal concentrations can be seen in Figures 4-21 to 4-25. When pH is altered the effects of increasing metal concentrations may be seen in Figures 4-26 to 4-34. The model predicts that as the metal concentration increases so will inhibition, thus the NMA will continue to drop until it is in effect zero. This however is not always the case in the results. The best example of this is copper at pH 9 (Figure 4-28). Copper at very low concentrations is very toxic (Figure 4-22); however at varying copper levels in a pH 9 solution copper toxicity is virtually halted. This is likely because at a certain point copper begins to drop out of solution as a metal phosphate hydroxide as described in Section 4.1 and shown in XRF experimentation. The solid metal is no longer available for complexation with the cell and any metal above the solubility point is dropped out of solution. Thus the metal in solution remains at the solubility point and toxicity is virtually fixed at that point, Figure 4-35. This phenomena is observed for many of the metals at many pH levels (Figures 4-23, 4-28, 4-29, and 4-30).

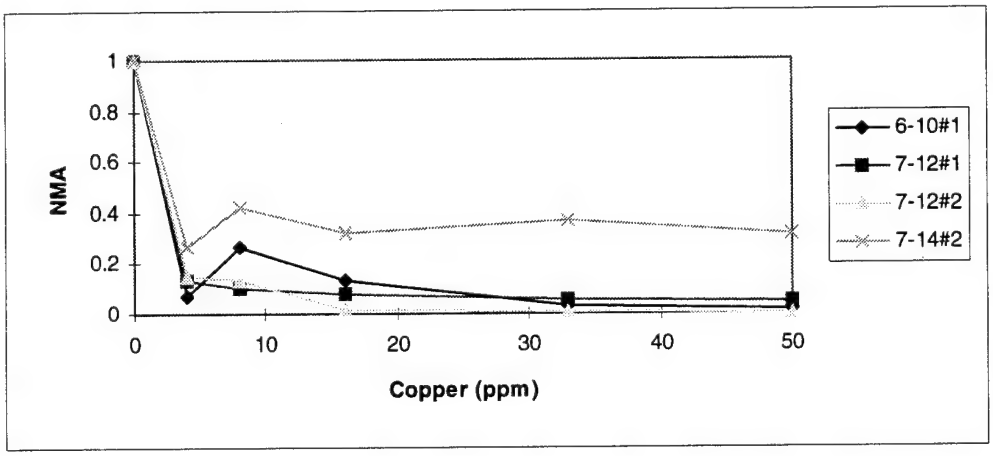


Figure 4-21
NMA as a function of Copper Concentration pH 6
Replicate experiments measuring NMA as a function of copper concentration

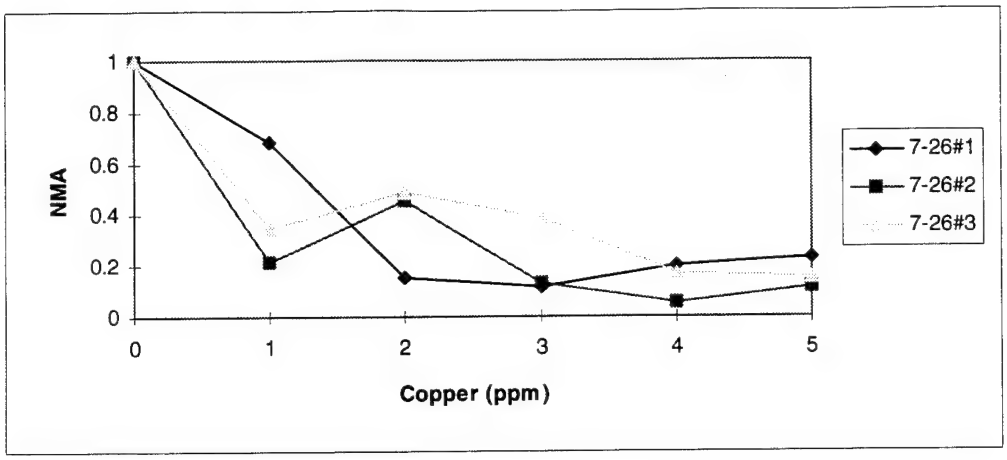


Figure 4-22
NMA as a function of Copper Concentration pH 6
Replicate experiments measuring NMA as a function of copper concentration

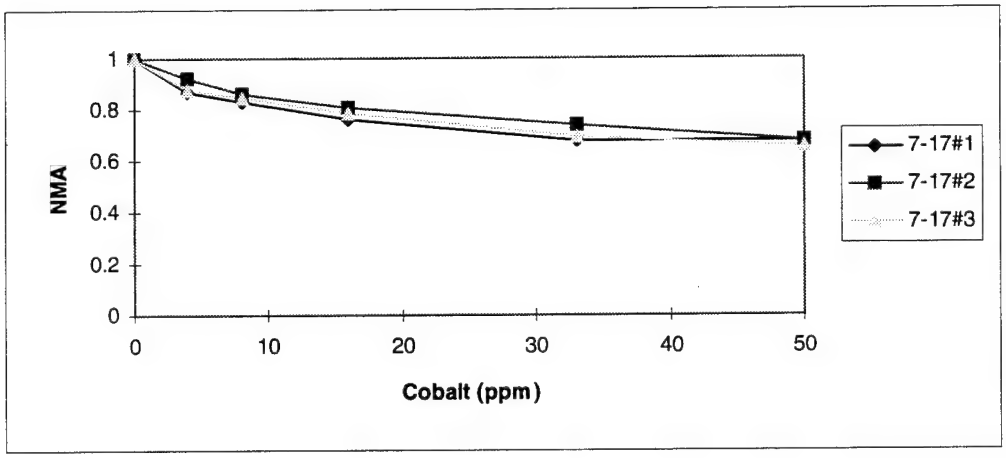


Figure 4-23
NMA as a function of Cobalt Concentration pH 6
Replicate experiments measuring NMA as a function of cobalt concentration

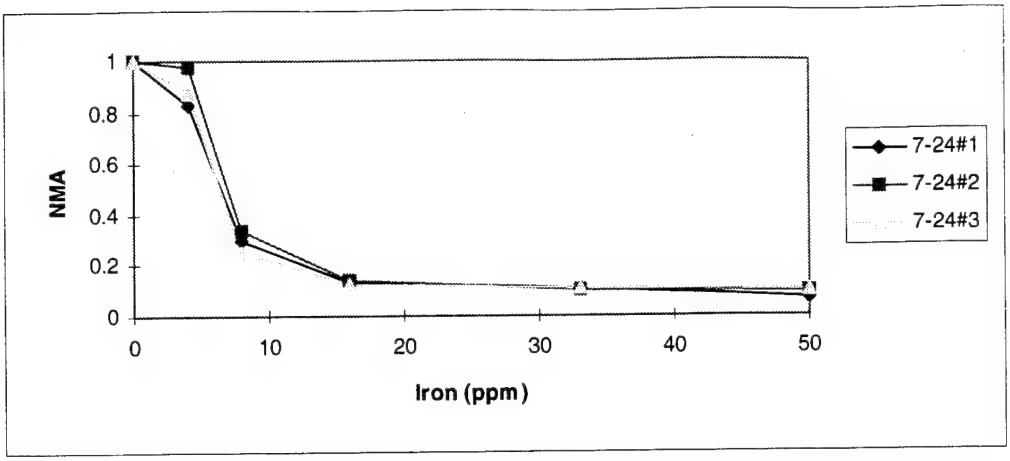


Figure 4-24
NMA as a function of Iron Concentration pH 6
Replicate experiments measuring NMA as a function of iron concentration

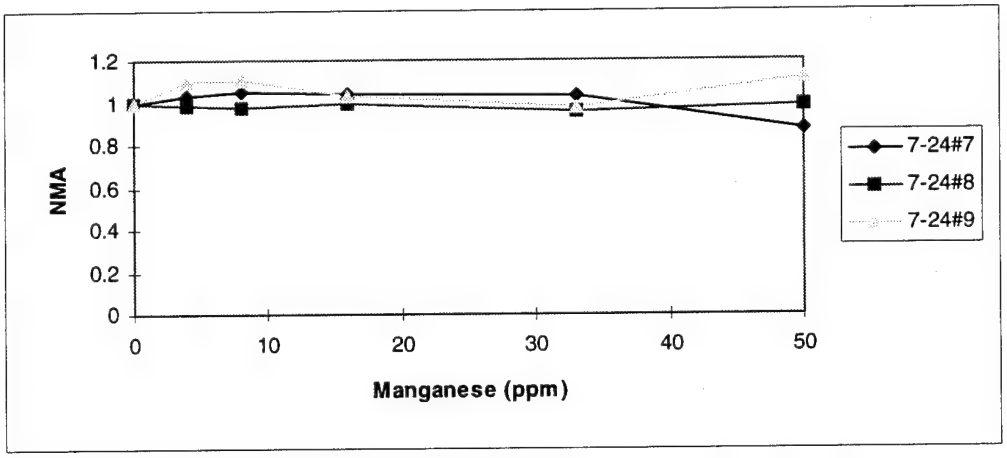


Figure 4-25
NMA as a function of Manganese Concentration pH 6
Replicate experiments measuring NMA as a function of manganese concentration

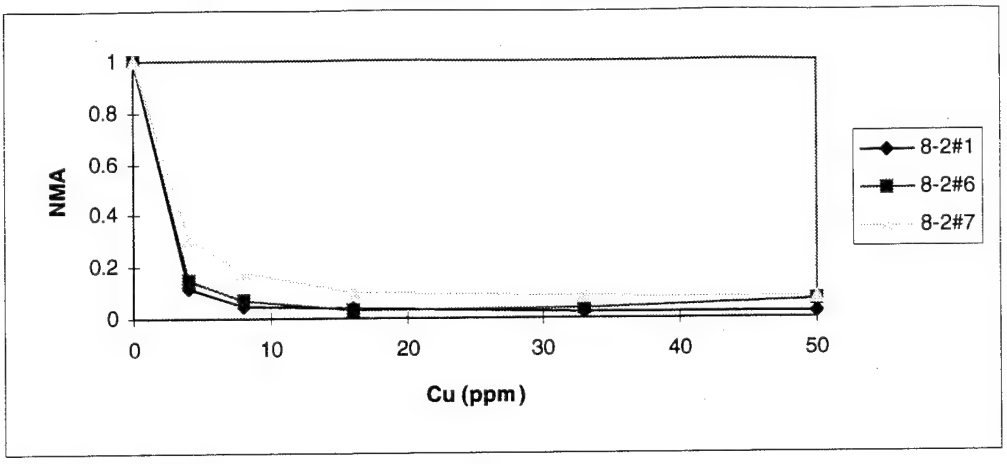


Figure 4-26
NMA as a function of Copper Concentration pH 5
Replicate experiments measuring NMA as a function of copper concentration

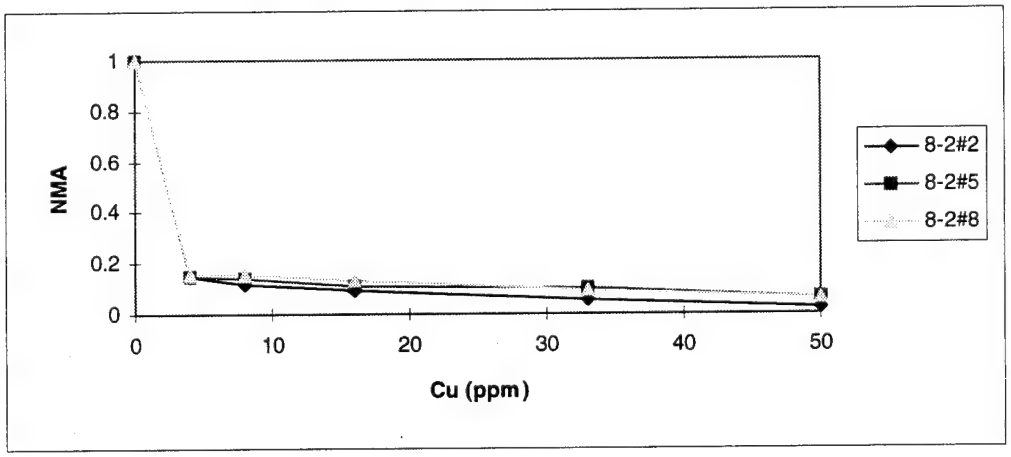


Figure 4-27
NMA as a function of Copper Concentration pH 7
Replicate experiments measuring NMA as a function of copper concentration

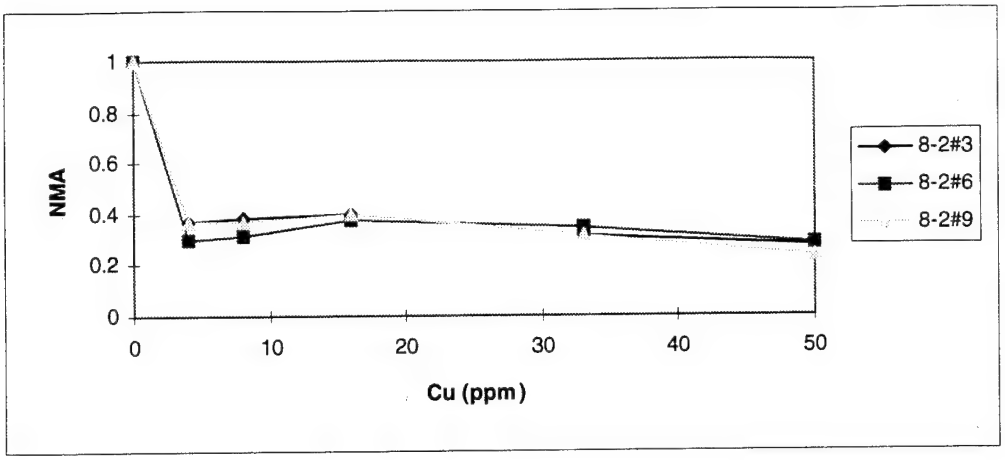


Figure 4-28
NMA as a function of Copper Concentration pH 9
Replicate experiments measuring NMA as a function of copper concentration

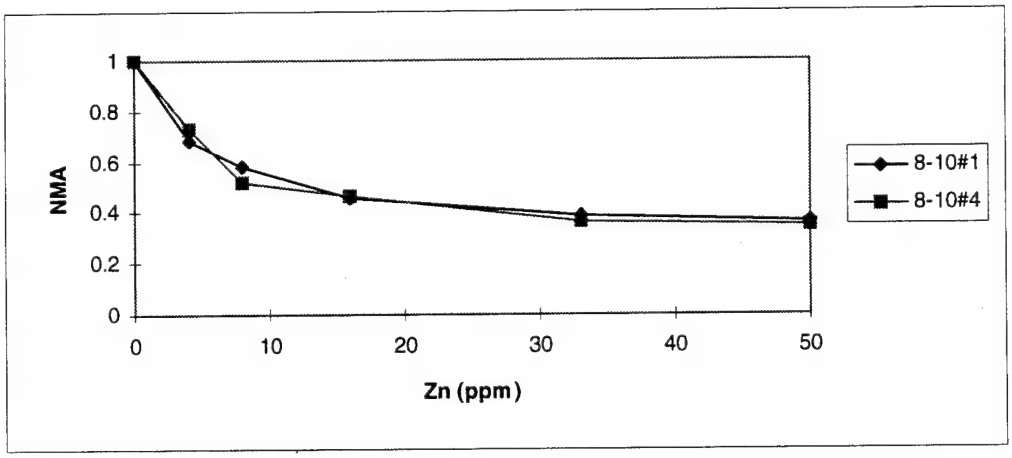


Figure 4-29
NMA as a function of Zinc Concentration pH 5
Replicate experiments measuring NMA as a function of zinc concentration

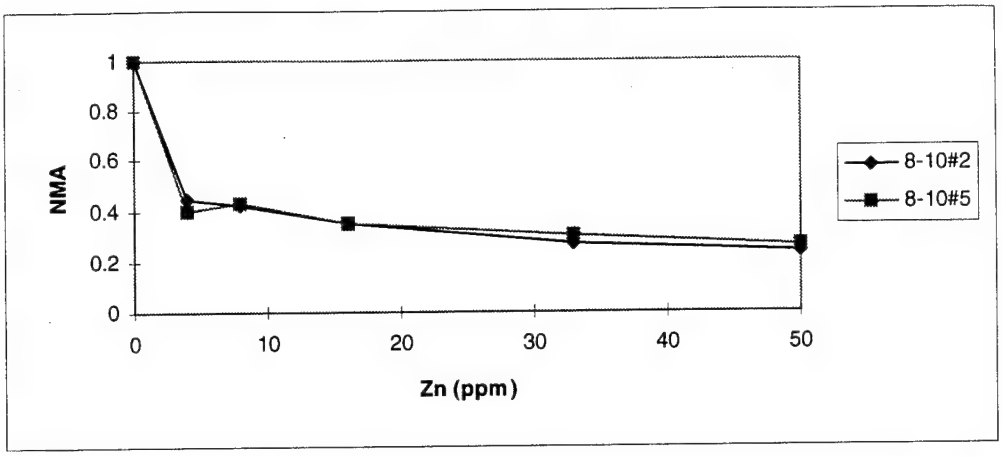


Figure 4-30
NMA as a function of Zinc Concentration pH 7

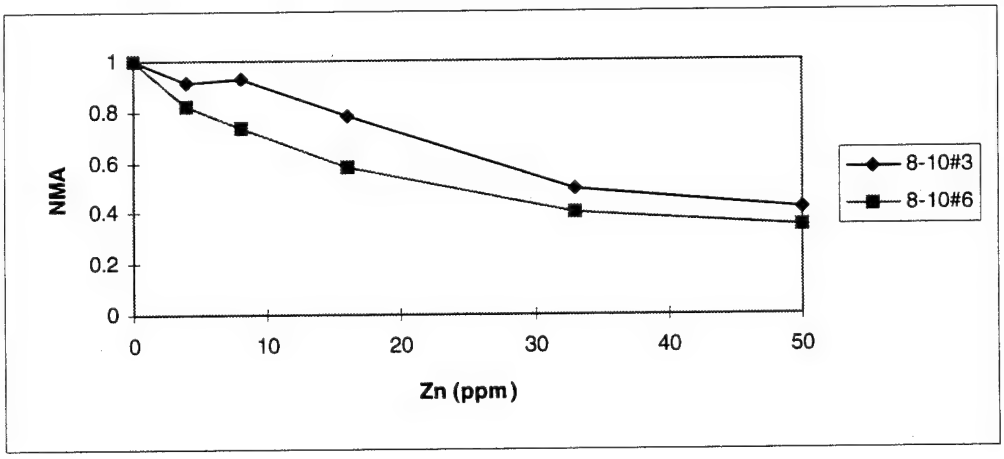


Figure 4-31
NMA as a function of Zinc Concentration pH 9
Replicate experiments measuring NMA as a function of zinc concentration

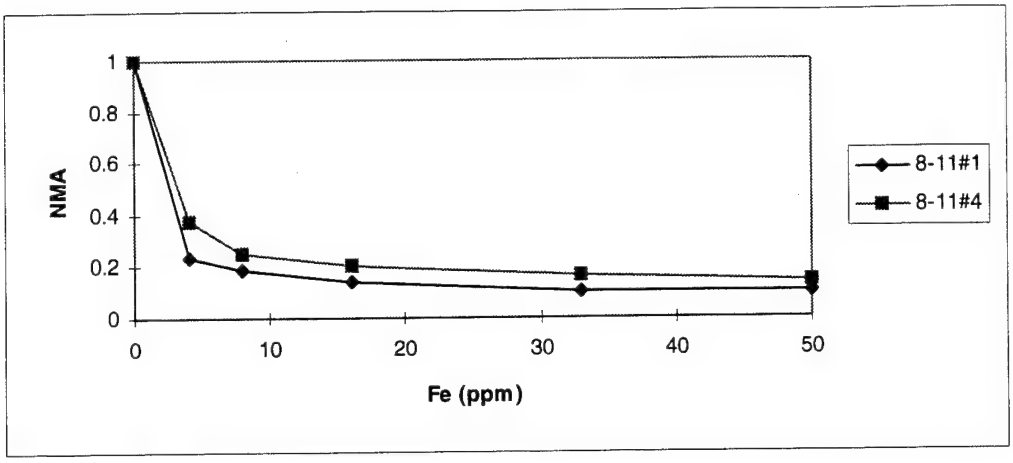


Figure 4-32
NMA as a function of Iron Concentration pH 7
Replicate experiments measuring NMA as a function of iron concentration

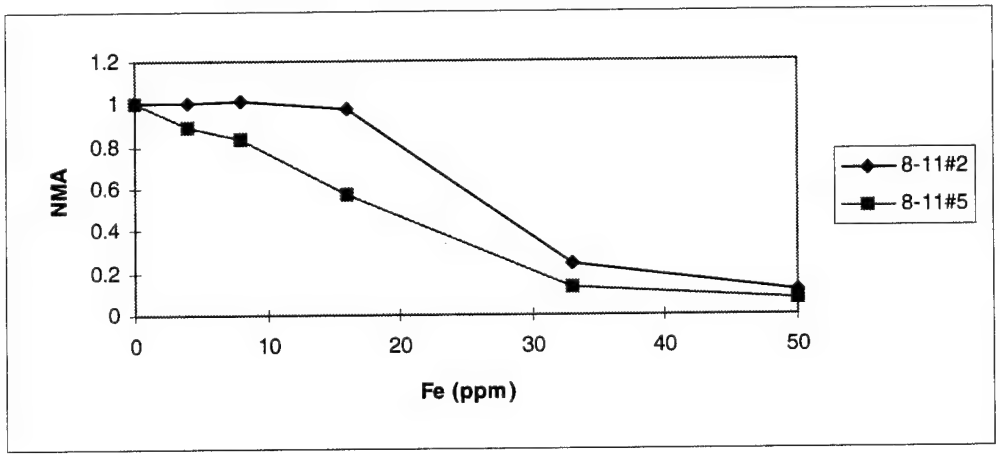


Figure 4-32
NMA as a function of Iron Concentration pH 7
Replicate experiments measuring NMA as a function of iron concentration

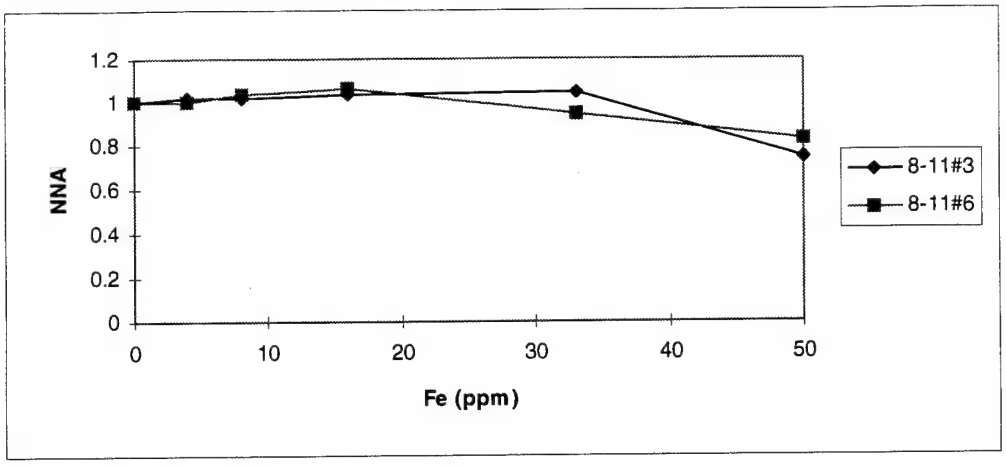


Figure 4-34
NMA as a function of Iron Concentration pH 9
Replicate experiments measuring NMA as a function of iron concentration

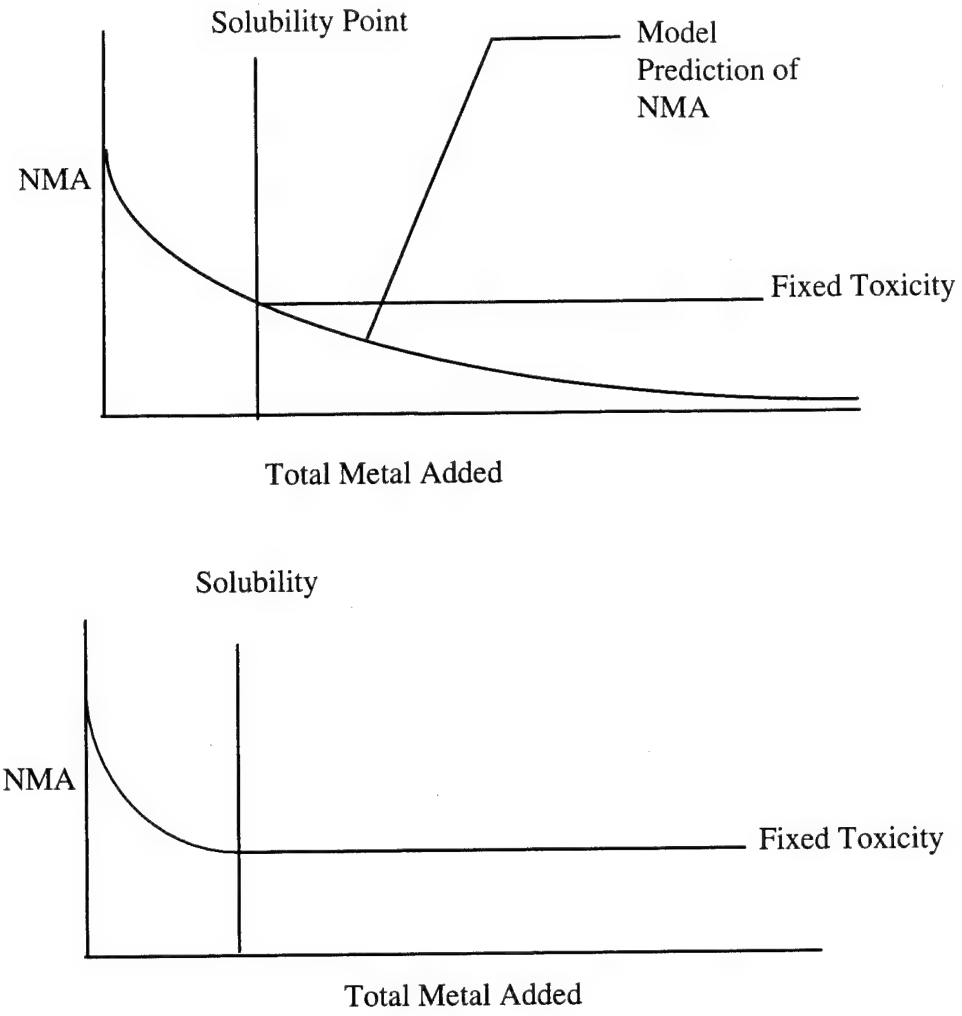


Figure 4-35
Prediction Model for Metabolic Inhibition with Precipitation
Inhibition increases with increasing metal concentration; however it does so at a decreasing rate until precipitation occurs

4.9 Effect of EDTA on the Metabolic Inhibition of Copper

Three separate sub-experiments were conducted with EDTA to determine its influence on metal inhibition. The purpose of these experiments was to determine if

EDTA could "shield" microorganisms from the effects of a metal. This would be further proof that it is the availability of metal that effects inhibition. If successful, it would provide additional information of an upper bound of the cell membrane ligand association constant. In addition the experiments will address whether the inhibition caused by the metal is reversible, if a metal is adsorbed on to a cell membrane and then removed will the inhibition be removed or is their damage already done.

The first experiment described in Section 3.5.7.1 examined the effect of increasing EDTA concentrations on the metabolic inhibition of copper. The results are presented in Table 4-17.

Table 4-17
Effect of Increasing EDTA Concentration on NMA

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Cu (ppm)	0	0	33	33	33	33
EDTA (ppm)	0	333	0	66	166	333
NMA	1.00	.964	.173	.131	.229	1.27

The results show a dramatic increase in the respiration rate of the microorganisms between 166 and 333 ppm EDTA concentration; however the increasing trend actually begins between 66 and 166 ppm. If EDTA is extremely effective at competing with cellular membrane components and aquatic ligands for metals then virtually all the copper will exist in a copper-EDTA complex. Let's look again at the results between 66 and 333 ppm EDTA for metal dosed microcosms this time looking instead at the concentration of copper and EDTA in molar terms, Table 4-18. At 166 ppm the number of moles of EDTA is approaching the number of moles of copper; it appears that EDTA is successful in competing with the microorganisms for the metal, thus there is much less

metal available to the cell for inhibition. At 333 ppm EDTA, there is more EDTA than copper, if EDTA has bound the vast majority of copper then no inhibition should occur; this is indeed the case.

The fact that the NMA is significantly greater than 1 indicates that either a portion of the population can use EDTA as a food source, or that there is another cation substance exerting inhibition over the microorganisms that is also chelated by the EDTA.

Table 4-18

	Sample 4	Sample 5	Sample 6
Cu (ppm)	33	33	33
Cu (M)	$5.2x10^{-4}$	$5.2x10^{-4}$	$5.2x10^{-4}$
EDTA (ppm)	66	166	333
EDTA (M)	$1.5x10^{-4}$	$3.8x10^{-4}$	$7.69x10^{-4}$
NMA	.131	.229	1.27

The EDTA is better at competing for the copper than any other ligand in the solution and its complexation constant is higher for copper than it is for any other cations in the solution. This means that when the molar concentration of EDTA is lower than copper all the EDTA is tied up as a copper-EDTA complex, thus the metal toxicity exerted by the cell shall be equivalent to the difference between the copper and EDTA concentrations. First a composition graph of the toxicity of copper was prepared by taking the average of NMA values from Figures 4-21 and 4-22 (Figure 4-36). The free copper concentration was then calculated for samples 4 and 5 from Table 4-36. The assumptions were made that all the EDTA was complexed as copper-EDTA and that this complex is not toxic. The results of the EDTA experiment were then compared to the averages from Figure 3-36. These results are presented in Table 4-19. The correlation

between observed inhibition and predicted inhibition is excellent indicating that the EDTA is indeed protecting the microorganisms from the toxic effects of the copper.

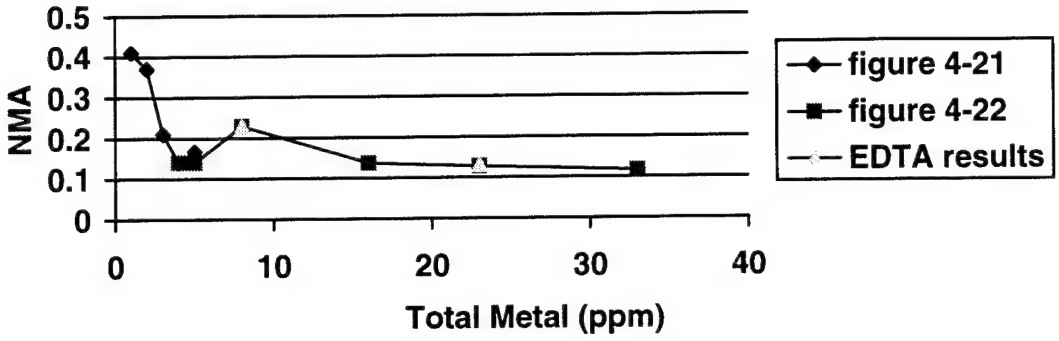


Figure 4-36
Compilation of toxicity between 1 and 16 ppm
from Figures 4-21 and 4-22

Table 4-19
Comparison of EDTA Observed Inhibition vs Predicted Inhibition from Previous Experiments

	Sample 4	Sample 5
Calculated free copper (ppm)	23	9
NMA observed	.131	.229
Predicted NMA from figure 4-31	.128	.23

The first experiment has shown that microorganisms may be protected from the effects of metals by the addition of EDTA or some other strong chelator if it is added in the right molar proportions. The two next experiments investigate whether metal ion may be "stolen back" from microorganisms once adsorbed onto the cell membrane and

whether or not the inhibition of these metals can be reversed. The results are presented in Table 4-20.

Table 4-20
Effect of EDTA on the toxicity of Copper

	initial rate (mgO_2/min)	Rate after Copper treatment (mgO_2/min)	Rate after EDTA treatment (mgO_2/min)	Difference in Metabolism rate (mgO_2/min)
Copper then EDTA	.585 .342 .217 .444	.007 .033 .047 .044	.039 .05 .058 .044	.032 .017 .011 0
EDTA and Copper together	.088 .176	.073 .199	- -	-.015 .023

The results show that the addition of EDTA does increase the metabolism of the microbial population; however it is not possible to say if this increase is due to a "reversal" of inhibition or the increased activity of a population of microorganisms that is adapted to EDTA as a food source. Although the microbial population was adapted to toluene as the sole source of carbon, a small quantity of EDTA was present in the Miracle-Gro solution; it is possible that a population of EDTA consuming microorganisms existed in this large and diverse microbial community. The fact that the increased metabolism is only a small fraction of the activity lost indicates that even if EDTA is capable of stealing metal back from the cell after adsorption the damage to the cell is already done.

V Conclusions

This chapter will summarize the results of the thesis and suggest areas for follow on research.

5.1 Summary of Results

In this thesis effort 11 different experiments were conducted investigating eight factors that could potentially effect microbial metabolic inhibition. In addition to the toxicity of the metals tested, an attempt was made to correlate the inhibitory effects of a metal to its microbial toxicity.

In general, the results showed that the inhibition of microbial respiration does not correlate with survival rate of metal-dosed microorganisms as measured by sterile plating techniques. In addition, results showed that metal inhibition or respiration is affected by many environmental factors such as pH, competitive cations, population size, nutrient levels, sodium chloride and EDTA. The following sections summarize the results and conclusions.

5.1.1 Effect of pH

The effect of pH on microbial inhibition was described in Section 3.5.2 and its results presented and discussed in Section 4.1. The results of the experiment were two fold: metals were rank ordered according to inhibition and the effect of pH on inhibition was determined. An increase in pH was found to increase inhibition; however, between pH 7 and 9 precipitation of the metals occurs and the microbial inhibition decreases.

5.1.2 The Effect of Magnesium on Toxic Metal Microbial Inhibition

The effect of magnesium on metal inhibition was described in Section 3.5.4 and the results presented in Section 4.2. The results showed that increasing magnesium concentrations decreased metabolic inhibition by the toxic metals. This information, when combined with metal adsorption x-ray data shows that as magnesium is increased NMA increases and the metal adsorbed to the microorganism decreases. The changes, both positive and negative, are approximately the same proportion as the concentration of magnesium is increased. The magnitude of change for each metal tested between magnesium treatment levels corresponded to the magnitude of the metal-phosphate ligand binding coefficient. This implies that replacing the inhibitory metal on the cell membrane of a microorganism by magnesium had the effect of reducing that metals inhibition.

5.1.3 Effect of Calcium on Toxic Metal Microbial Inhibition

The effect of magnesium on metal inhibition was described in Section 3.5.4 and the results presented in Section 4.3. This experiment is very similar to the magnesium experiment except the competition cation is calcium. One would expect similar results, but this is not what occurred. Inhibition was decreased as calcium was increased from 0 ppm to 50 ppm total calcium, but increased again between 50 ppm and 100 ppm total calcium concentration. This result was not substantiated with metal adsorption data due to time constraints. The rank order of percent change in NMA corresponded to a rank ordering of the metal-hydrogen phosphate ligand binding coefficients; it was postulated that metals were being displaced from the aquatic phosphate. These metals then became available to the microorganisms to cause increased toxicity.

5.1.4 Effect of Microbial Population Size on Inhibition

The effect of magnesium on metal inhibition was described in Section 3.5.5 and the results presented in Section 4.4. As expected, the inhibition of copper and cobalt decreased slightly with the increase in population size. Zinc, however, showed significant increases in inhibition as the population size was increased. This result was not able to be explained in this thesis effort. It might be examined further in future thesis efforts.

5.1.5 Effect of Nutrient Levels on Toxic Metal Inhibition

The effect of nutrient level on metal inhibition was described in Section 3.5. and the results presented in Section 4.5. In this experiment, the level of nutrients was varied and the inhibition noted. This experiment failed to account for the change in pH that occurred as more ammonium dihydrogen phosphate was added to the dilution water. Thus any reduction in inhibition that occurred in this experiment could not totally be attributed to the nutrients but to the pH and nutrient effects. The magnitude of change for copper and zinc inhibition, however, was much larger than for a change in pH alone. This indicates that the nutrients do have the ability to abate the effects of metal inhibition. In addition, the magnitude of NMA increase for each metal as nutrient levels were increased corresponded to magnitude of the metal-phosphate ligand binding indicating increased competition with phosphate ligands causes the microorganism to have less metal available to them for binding. The lack of availability metal due to competition with other ligands leads to lower inhibition.

5.1.6 Effect of Sodium Chloride on the Metabolic Inhibition of Toxic Metals

The effect of sodium chloride on metal inhibition was described in Section 3.5.8 and the results were discussed in Section 4.8. Sodium chloride was found to have differing effects on metal inhibition. It was found to increase the inhibition of copper 210% while decreasing the inhibition of cobalt 25%; zinc showed change in inhibition as sodium chloride levels were increased. The changes in inhibition were unexpected due to chlorides low ligand binding strength with the metals as compared to other ligands in the dilution solution and sodium's ligand binding strength is much lower than the metals tested.

5.1.7 Investigating the Lethality of Metals

This experiment was discussed in Section 3.5.4 and the results presented in Section 4.7. This experiment showed that a healthy, diverse population existed in the innoculum population, this implies that inhibition effects are the culmination of the inhibition effects of several microbial species including both heterotrophic bacteria and fungi. The results of this experiment did not correlate well with the results of the inhibition experiment indicating that NMA might not be an indicator of microorganism viability after an encounter with metals.

Two conclusion were drawn from the results of this experiment when compared to the NMA experiments. First, the ratio of copper treated colonies to control colonies did correlate well to NMA. This indicates that copper does indeed seem to kill most of the microorganisms that come into contact with it. Zinc on the other hand, has a much higher zinc treated colony to control colony ratio than NMA. This indicates that zinc is

inhibitory and that microorganisms dosed with zinc are not killed but instead inhibited by the presence of the metal. Manganese produced a high mortality rate even though it virtually showed no metabolic inhibition; however, there were not replicates of this experiment and it is possible that this result is an out lier. The correlation between metabolic activity and microorganism viability could definitely be investigated further.

5.1.8 Effect of Varying Metal Concentration on NMA

In this experiment the inhibition of a metal as a function of its total metal concentration was investigated. This experiment was discussed in Section 3.5.1 and the results presented in Section 4.8. The conclusions from this experiment were two fold. First, metal inhibition increases with metal concentration; however, it does so at a decreasing rate. The increase in inhibition should theoretically increase with increasing total metal concentration to 100% inhibition; however, this was not found to be the case. Increasing metal concentration did cause increased inhibition, but only to a certain point. It is believed that at or above this point all extra metal that enters the microcosm is dropped out of solution as a metal phosphate hydroxide complex. Metal that precipitates out of solution would be unavailable to the microorganisms thus causing a plateau in metal inhibition at the level at which precipitation occurred.

5.1.9 Effect of EDTA on the Metabolic Inhibition by Copper

EDTA is a strong organic chelator that is capable of very strongly binding metal ions. A series of short experiments was conducted to determine the interactions of EDTA and copper on the metabolic activity of microorganisms. The experiments were described

in Section 3.5.7 and the results were presented in Section 4.9. The results of the experiment show that EDTA when added to a microcosm in the proper molar proportions before copper addition can eliminate the inhibition effects of copper. The elimination in inhibition was likely because of a much higher copper-EDTA association constant than the copper-cell association constant. This puts a bound on the copper cell ligand association constant at log (K)<20.5. Increasing the nutrient concentration especially phosphate (Section 4.5) had an effect on inhibition but not nearly as substantial as that of EDTA. This leads to the conclusion that the copper microorganism ligand binding coefficient can be bounded by the copper-phosphate association constant (log(K)=16.5) and the copper-EDTA ligand association constant with the value most likely closer to phosphate than EDTA.

An attempt was made to remove copper from microorganisms that had already been dosed with copper. Although NMA showed a slight increase with the addition of EDTA, it could not be concluded that the removal of copper by EDTA reduced inhibition. Instead the meager recovery seemed to indicate that once the copper bound its self to a cell the damage was either permanent causing no change when EDTA removed the copper or the copper was not removed from the cell membrane, because the copper was no longer accessable to the EDTA.

5.1.10 Summary

The overriding concern in the inhibition of microorganisms by metals seems to be competition. The competition between metals for binding ligands on the cell wall of microorganisms was found to be important. In addition, competition between ligands on

the cell membrane and organic and inorganic ligands in the aquatic environment also seems to play a major role in metal inhibition. Metal availability plays a role in metal inhibition; if a metal has precipitated out of solution it was found to be no longer available to cause inhibition. Finally the magnitude of the effect of an environmental factor was often correlated to the metal-phosphate ligand association constant.

5.2 Follow on Research

There are several areas ripe for follow on research. Some involve the use of a single species of microorganisms, others involve the use of different analytical techniques to determine metal toxicity and metal adsorption. Still others involve the use of more toxic hazardous substances that are also likely to be in an industrial waste stream. Some follow on research ideas are as follows:

(1) One area of possible research involves the testing of a pure microorganism culture that is of possible interest to the Air Force. The Environics Directorate at Armstrong Labs has several such species.

(2) Further experimentation as to the cause of toxicity could be investigated. The use of electron microscopy would be useful in determining if the metals are penetrating the cell membrane to cause toxicity.

(3) The effect of metals on microorganisms could be modeled using Monod growth kinetics. This could be useful in the design of bioreactors to ensure that microorganism wash out does not occur due to slowed growth kinetics

(4) Some of the areas of interest that were identified in this thesis could be further studied. For example the increase in toxicity of zinc with increased population size and

the effect of sodium chloride and calcium experiments could be carried further in an attempt to explain there phenomena. The results of the research can be used to improve the accuracy and usefulness of a cellular model.

5.3 Parting Thoughts

This research represents a first attempt at studying the metal toxicity in microorganisms and was one of the first experimental bioremediation efforts attempted at AFIT. As such, there are many lessons to be learned and new ground to be paved. In the future, a better method of culturing the microorganisms will have to be devised. There exist several commercial incubation devices that could be obtained to culture mixed or pure microorganism cultures. In addition, more experience with the growth medium is needed, Miracle-Gro, although adequate, was not an ideal growth medium. There are several sources that present microbial culturing solutions, but AFIT still does not have the proper materials necessary to make these solutions. Additionally, metal precipitation was a problem in this thesis effort; future experiments will have to work to eliminate this problem. This might mean working at lower pH's or lower nutrient levels to prevent the metals of interest from precipitating out of solution.

This thesis effort represented a good first step to define the problems associated with researching metal toxicity; future thesis students will be able to use this research to scope their effort and as a good background effort.

The information derived in this thesis can be of use to environmental engineers that may be attempting to design biofilters or bioremediation of mixed waste sites. When the engineer is designing a remediation system, certain factors can be controlled with in

cost constraints. For example, the addition of nutrients may be necessary to aid in bioremediation; however, the addition of a little more phosphate may act to precipitate some of the metals out of solution. This would make the toxic metals unavailable to the microorganism, increasing the bioremediation rate of the organic hydrocarbon. If an opportunity exists to alter the pH, an engineer might lower the pH to decrease the toxic effects of a metal or increase the pH to precipitate the metal out of solution.

There seem to be no hard and fast rules to dealing with metal toxicity, thus there will always be a need for bench scale testing of any remediation effort. However, the trend discovered in this thesis might help to reduce the number of options tested and better optimize the remediation of mixtures of organic and inorganic substances.

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Vita

Captain Scott Hansen was born on 12 July 1968 in Edison New Jersey. He graduated from the Peddie School in Hightstown New Jersey in 1986. He attended college at Lehigh University in Bethlehem Pennsylvania, receiving a Bachelor of Science in Mechanical Engineering and a reserve officer commission in June of 1990. In March of 1991 he entered active duty; his first assignment was to Griffiss Air Force Base in New York where he served as a heating, ventilating, and air conditioning design engineer and chief of the Maintenance Engineering Section. He served at Griffiss AFB until May 1994 when he entered the Air Force Institute of Technology.

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This research was a general phenomenalistic overview of the effects of metals on the respiration rates of a toluene selected microbe culture. The metals studied were copper, zinc, cobalt, manganese and iron. Relative toxicity, Cu>Zn>Co>Fe>Mn, corresponded to ligand binding strengths. Toxicities were found to be pH dependent; it increased with increasing pH and was shown to correlate to an increased adsorption of metal onto the cell. Zn, Cu, and Co toxicity was shown to decrease with increasing magnesium concentrations. This decrease was linked to increased magnesium adsorption and decreased metal adsorption. The magnitude of effect was related to Mg competitiveness. Sterile plating techniques were used to determine metal lethality. Results did not correspond to respiration results; however, it was demonstrated that copper's effect was lethal while zinc's was inhibitory. Increasing metal concentrations cause toxicity to increases at a decreasing rate only so long as the metal remains in solution. Metals that precipitate are no longer available to adsorb to the cell and do not affect toxicity. Toxicity was shown to be related to metal availability and how well it competed with other cations. Toxicity also depended on cell-ligand competition and how strongly the metal bound itself to the cell.

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